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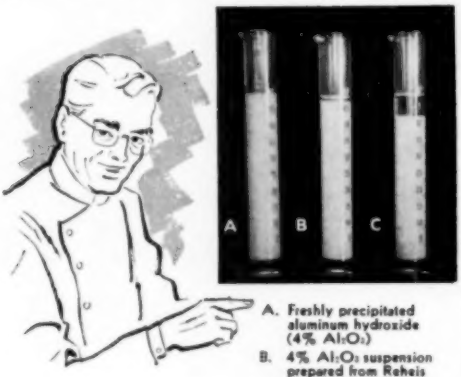

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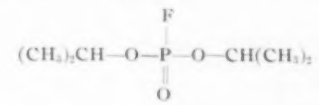
ALSEROXYLON.—Rauwiloid (Riker).—A fat-soluble alkaloidal fraction extracted from the root of *Rauwolfia serpentina* (Benth), containing reserpine and other nonadrenolytic amorphous alkaloids.

Actions and Uses.—Alseroxylon has the action of the sedative-antihypertensive-bradycrotic alkaloids of *Rauwolfia serpentina*, of which reserpine is among the most potent. It has the same uses and limitations as the whole root, rauwolfia, and its chief component alkaloid, reserpine. (See monograph on reserpine.)

Dosage.—Alseroxylon is administered orally. For adults the average oral dosage is 2 to 4 mg. daily. Higher oral dosage may be required for sedation of gross psychotic disturbances than for anxiety-tension syndromes associated with mild, labile hypertension or as an adjunct to more potent hypotensive agents in essential hypertension. (Orally, 1 mg. is approximately equivalent to 0.2 mg. of reserpine.)

ISOFLUROPHATE, U.S.P.—Floropryl (Merck, S. & D.)—$C_6H_{14}FO_3P$.—M.W. 184.15.—Diisopropyl fluorophosphate.—The structural formula of isoflurophate may be represented as follows:



Actions and Uses.—Isoflurophate, a parasympathomimetic agent, acts chiefly by irreversible inhibition of the enzyme, cholinesterase. The anticholinesterase activity of isoflurophate is more prolonged than that of physostigmine and similar drugs; thus it is more convenient for maintaining miosis in the treatment of glaucoma, with fewer instillations. Systemic administration of the drug is contraindicated because this produces a profound reduction of plasma cholinesterase that is associated with prolonged and sometimes serious effects of generalized parasympathetic stimulation, including salivation, lacrimation, intestinal cramps, urge to urinate and defecate, shock, and collapse. In addition to its widespread cholinergic effect, large systemic doses of the drug also produce paralysis of autonomic ganglions that resembles the systemic action of nicotine. When the drug is instilled locally in the eye, plasma cholinesterase is reduced only slightly and seldom is accompanied by systemic effects. Isoflurophate produces a powerful and relatively prolonged miotic action and spasm of the muscles of accommodation caused by the local antagonism of cholinesterase. It is used by corneal instillation to reduce intraocular tension in the treatment of aphakic glaucoma and chronic noncongestive (wide angle) glaucoma, for preoperative control of glaucoma, and as an antidote against the harmful action of atropine on preglaucomatous and glaucomatous eyes. Many ophthalmologists consider the drug to be contraindicated in acute congestive (narrow angle) glaucoma because of the danger of increasing intraocular pressure.

Isoflurophate has a tendency to produce ciliary spasm and, rarely, a rise rather than a reduction in intraocular tension. This latter response probably can be forestalled if tonometric examinations are made hourly after the initial use of the drug, since instillations of 1 or 2% epinephrine solution at 5 minute intervals, following detection of increased pressure, may be successful in preventing any further rise. The ciliary spasm that is produced at times by isoflurophate may cause brow pain and headache that may be minimized and at times eliminated by the use of smaller amounts of the drug. Local sensitivity to the drug or the solvent in which it is applied may occur, but such reactions are infrequent. Resistance to its tension-lowering effect may develop in some patients after several months. Retinal detachment has been reported in high myopia produced by isofluorophate, but the incidence appears to be no greater than that following the use of other commonly employed miotics. In case of accidental systemic administration, the concomitant injection of atropine sulfate to combat cholinergic action and magnesium sulfate to combat nicotinic action may be indicated; however, unless the systemic dose is sufficiently high to produce ganglionic paralysis, 0.4 to 0.6 mg. of atropine sulfate alone, parenterally, usually is adequate to overcome the parasympathomimetic effects of the drug in such emergencies.

Dosage.—Isofluorophate is administered locally in the eye by corneal instillation of a 0.1% solution in vegetable oil. The usual dosage is one to three drops of such solution in the affected eye every 8 to 72 hours. If possible, instillation should be made at night before retiring so that blurring of vision will be less disturbing.

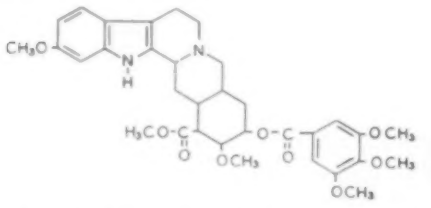
RAUWOLFIA.—Raudixin (Squibb).—The powdered whole root of *Rauwolfia serpentina* (Benth).

Actions and Uses.—Rauwolfia produces the sum of the actions of the total alkaloids contained in the whole root. The component alkaloids exhibit the sedative-antihypertensive-bradycrotic action characteristic of reserpine, the latter of which accounts for approximately 50% of their total activity. The whole root contains only insignificant amounts of

yohimbine and other adrenolytic alkaloids. Thus, rauwolfia is useful for the same purposes and with the same precautions as for reserpine. See the monograph on reserpine.

Dosage.—Rauwolfia is administered orally. For adults the average oral dosage of the powdered whole root is 200 to 400 mg. daily, administered in two divided doses. Larger doses may be required for sedation of gross psychotic disturbances than for anxiety-tension states associated with mild, labile hypertension or as an adjunct to potent hypotensive agents in essential hypertension. Orally 200 to 300 mg. of powdered whole root is equivalent to 0.5 mg. of reserpine.

RESERPINE.—Crystoserpine (Dorsey)—Eskaserp (SK & F)—Rau Sed (Squibb)—Reserpoid (Upjohn)—Roxinoid (Sharp & Dohme)—Sandril (Lilly)—Serfin (Parke, Davis)—Serolfia (Ascher)—Serpanray (Panray)—Serpasil (Ciba)—Serpate (Vale)—Serpen (Haag)—Serpiloid (Riker)—Serpine (Pitman-Moore).—$C_{33}H_{40}N_2O_9$.—M.W. 608.70.—An ester alkaloid isolated from the root of certain species of Rauwolfia. The structural formula of reserpine may be represented as follows:



Actions and Uses.—Reserpine is considered one of the most potent of the Rauwolfia alkaloids, which exhibit a sedative action and antihypertensive effect accompanied by bradycardia. Its antihypertensive effect is variable and ineffective in some patients. Studies in experimental animals indicate that the drug acts on the central nervous system, producing a tranquilizing nonhypnotic sedation, ptosis of the eyelids, sinus bradycardia, increased secretory and motor activity of the gastrointestinal tract, and a variable lowering of mean arterial blood pressure. The latter effect is attributed to a decrease in peripheral vasoconstriction apparently induced by central action on the vasomotor mechanism and is not associated with a significant alteration of cardiac output or renal plasma flow. Some effects of the drug suggest an action on the autonomic nervous system, although it does not exhibit either peripheral adrenolytic or peripheral parasympathomimetic activity and it does not block autonomic ganglions. Its observed effects are presumed to result from a central partial suppression of sympathetic predominance near the level of the hypothalamus where autonomic nervous functions are integrated. In contrast to barbiturates, reserpine produces less somnolence and does not alter significantly the electroencephalogram, depress the cortex, or prevent stimulant-induced convulsions.

The dose-response curve of reserpine is rather flat, although large doses may be associated with increased side-effects. After a single intravenous dose, the onset of sedative action of the drug usually occurs within about 30 minutes and the onset of antihypertensive effect usually begins within an hour. After intramuscular injection, the onset of these effects may appear slightly later in the same subjects. The maximum sedative and antihypertensive effect usually is reached within about 4 hours, the sedative action may persist longer (up to 12 hours) than the antihypertensive action (up to 10 hours) after intramuscular injection. The variations in time of onset and duration of action appear to be more a function of dosage and individual responsiveness than the route of parenteral administration. With daily oral administration the effects of the drug usually are not fully manifest for several days to 2 weeks and may persist for as long as 4 weeks after oral medication is discontinued. Tolerance to the drug does not develop with continued administration.

Reserpine is useful chiefly for its tranquilizing sedative action in the symptomatic management of patients with anxiety or tension psychoneuroses as such or as an accompaniment to somatic disorders and of patients with chronic psychoses involving anxiety, psychomotor hyperactivity, or compulsive aggressive behavior. It is useful also for the management of mild, labile hypertension associated with anxiety and emotional factors and, in conjunction with potent hypotensive agents, for the management of essential hypertension and hypertension associated with toxemia of pregnancy. The use of reserpine alone is considered to be of little value for severe or fixed hypertension, but it is useful to enhance or prolong the action of potent hypotensive agents to reduce dosage and side-effects. It probably should not be used with barbiturates in eclampsia or other conditions in which profound sedation would be undesirable. The value of reserpine for routine use as a prophylactic against progressive vascular changes in patients with early hypertension has not been established. The exclusive use of reserpine in severe hypertension also may be considered dangerous because it may create a false sense of security at the expense of more effective measures.

Higher doses of the drug (administered orally and parenterally) usually are required in the management of grossly disturbed psychoses than in anxiety-tension states where the oral route usually is adequate. In both types of patients the drug must be administered for a period of 1 (usually) or 2 weeks before the optimal level of dosage can be determined. The tranquilizing effect of the drug may make the psychotic patient more accessible for concurrent psychotherapy; however, in the treatment of very disturbed psychotic patients, the effect of the drug should be carefully observed to avoid the development of an oblique attitude of indifference or detachment toward the underlying emotional conflicts. It should be realized that unless dosage is carefully adjusted the drug may induce a paradoxical form of anxiety and an adverse reactive depression. In chronic psychoses the drug does not appear to alter the basic psychopathological state.

Reserpine has a rather low toxicity but may be associated with undesirable or toxic side-effects especially at the high dosage levels. Side-effects are more likely to occur in hypertension when the dosage of the drug, which does not have a pronounced anti-

hypertensive effect, is increased in an effort to obtain a greater response. The side-effects commonly include nasal stuffiness, weight gain, and diarrhea and, less frequently, dryness of the mouth, insomnia, nervousness, paradoxical anxiety, increased gastrointestinal activity, chronic fatigue or sense of weakness in the extremities, muscular aching, nightmares, and agitated or paranoid depression. Skin eruptions occur rarely and peptic ulceration is a rare complication. Epistaxis also has been encountered. A paralysis agitans-like syndrome has been observed; this is reversible and apparently results from excessive dosage. Postural hypotension may occur occasionally after parenteral administration, but this rarely occurs after oral administration except with large dosage.

Parenteral therapy with reserpine alone is indicated in mild hypertension only when oral administration is not feasible. When relatively large doses are required, as in chronic psychoses, intramuscular injection is indicated in conjunction with oral medication; oral administration is best suited for use in conjunction with potent hypotensive drugs in chronic, severe hypertension. Because of the slow and variable antihypertensive effect, parenteral injection of reserpine alone is not suitable for the management of hypertensive crises.

Dosage.—Reserpine is administered orally and by intramuscular or intravenous injection. The usual oral dosage for adults in the treatment of mild, labile hypertension is 0.25 to 1 mg. daily in two or three divided doses. In some cases as little as 0.05 mg. or as much as 1.5 mg. daily may be required. As an adjunct to more potent hypotensive agents in severe hypertension, 0.25 to 0.5 mg. daily is usually adequate. In anxiety or tension psychoneuroses, the daily dosage is 0.5 to 2 mg. In disturbed psychotics an oral dosage of 3 to 5 mg. daily is usually employed in conjunction with intramuscular injection of 5 to 10 mg. daily, depending on the response to the drug.

SODIUM RADIO-CHROMATE (Cr^{51}).—Anionic hexavalent radioactive chromium (Cr^{51}) in the form of sodium chromate ($Na_2Cr^{51}O_4$). The radiochromium (Cr^{51}) has a half-life of 26.5 days. It disintegrates primarily by K capture, with transmutation to vanadium and emission of x-rays.

Actions and Uses.—The presence of a gamma ray of 0.32 mev energy in the radiation emitted by radiochromium makes sodium radio-chromate (Cr^{51}) useful as a biological tracer. When mixed with whole blood, the divalent chromate anion (CrO_4--) readily diffuses through the wall of the red blood cells and inside the cells is reduced to the trivalent chromic cation ($Cr+++$), which then firmly associates with the globin portion of their contents. Because the chromic form of the isotope apparently is not capable of passing the cell wall barrier and thus cannot be reutilized by intact cells, erythrocytes can be tagged by exposure to the diffusible chromate anion for determinations of circulating red cell volume and studies of red cell survival time. The isotope also can be employed for the determination of whole blood volume (red cell mass and plasma volume), used either alone or in conjunction with radioactive chromic chloride ($Cr^{51}Cl_3$). The radioactive chromic cation released by destruction of tagged erythrocytes readily combines with plasma proteins; however, the radioactivity of the plasma is lost more rapidly than that of the cells so that neither form of the isotope interferes with the other when used alone or simultaneously for such studies.

To tag erythrocytes, a sample of the patient's blood or of donor blood is mixed with a solution of sodium radio-chromate (Cr^{51}) and allowed to stand until the isotope diffuses into the cells. The unbound chromium (in the plasma) is then either reduced by addition of ascorbic acid or removed by separation and washing of the cells. The treated blood or a suspension of the separated cells is then injected into the circulation, and sufficient time is allowed for complete in vivo mixing before samples are taken for scintillation counting. Red cell or whole blood volume is estimated by the radioisotope dilution method. Red cell survival time may be studied by a similar technique with samples taken at appropriate intervals and corrections made for decay in gamma radiation. Consistently lower values for whole blood volume are obtained by the radioactive chromium method than by the dye method. Normal mean values for whole blood volume obtained by the isotope method are 65.6 ± 5.95 cc./Kg. of body weight, or 2.49 ± 0.28 liters per square meter of body surface.

Sodium radio-chromate (Cr^{51}) has not been shown to produce any significantly deleterious effects on normal erythrocytes, either from the standpoint of radioactivity or the amount of metallic chromium present. Because the bond that is formed with the cells is stable, the isotope has the advantage of permitting leisurely conducted radioisotopic studies of erythrocytes, including rechecks up to 24 hours, without correcting for loss of radioactivity. In studying red cell survival it eliminates the necessity for using donor cells and thus is free from the danger of incompatibilities of blood type or of transmission of the virus of serum hepatitis.

Dosage.—Sodium radio-chromate (Cr^{51}) is used in the form of a solution suitable for intravenous injection that first is mixed with a sample of 40 or 50 cc. of whole blood withdrawn from the patient or from a compatible donor. The dose is based upon the amount necessary to tag 80 to 90% of the erythrocytes in the sample of whole blood and ranges from 75 to 125μc (microcuries) with conventional techniques. Doses as low as 10 to 15μc have been used with special techniques for determinations of whole blood volume. After it is mixed with the appropriate dose, the sample should be allowed to stand for 15 to 60 minutes to achieve adequate tagging. After appropriate reduction or removal of the unbound portion of the isotope, the treated sample or separated suspension of erythrocytes is then injected and allowed to mix with the circulating blood before samples are withdrawn for counting.

As the tagged cells are very stable, repeat doses are seldom indicated. In conditions such as severe hemolytic anemia where red blood cell survival studies may be indicated before, during, and after treatment, and where repeat doses are necessary because of rapid destruction of red blood cells, the number of repeat doses should be kept at a minimum and the total dosage should not exceed 390 μc in the body at any one time.

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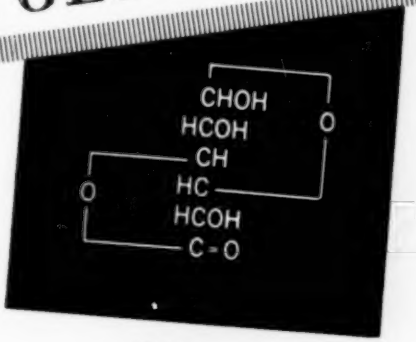

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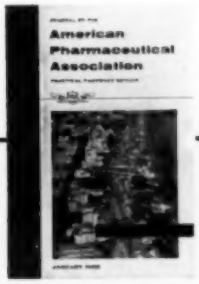


A Preview of Advertising

In the May 1956 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▶ "Specific against coccic infections" headlines **Abbott's** advertisement of its antibiotic, *Filmtab* **Erythrocin Stearate;** and for its antianemia therapeutic, *Filmtab* **Iberol,** the maker emphasizes that this oral hematinic's *iron-plus* characteristic "makes the difference."

▶ **Mannitol, Polyoxyl 40 Stearate, Polysorbate 80,** and **Sorbitol** bow as "N.F. and U.S.P. Pharmaceutical Aids of **Atlas.**"

▶ **Vaseline** sterile petrolatum gauze, $^1/_2$" × 72" . . . in disposable plastic tubes, is the subject of **Chesebrough-Pond's** announcement.

▶ **Azo Gantrisin**—for urinary infections—is pointed up by **Hoffmann-La Roche Inc.**

▶ Almost superfluous is the phrase, "tested and proved" authored by **Hynson, Westcott & Dunning** for **Mercurochrome.**

▶ The **Lederle Laboratories'** announcement graphically delineates a number of the maker's *prescription department specialties.*

▶ By adding vitamin B_6 and nicotinamide, **Lilly** has increased the *efficacy* of **'Homicebrin'**—but *not* its price.

▶ **Salcort,** product of the **S. E. Massengill Company,** is described as a "judicious" combination of salicylates and cortisone which results in "a much more favorable reaction in arthritis than with either alone."

▶ *Sig:* "Mark your store as prescription-minded pharmacy—and, at the same time, guide customers to your Rx department—with **Owens-Illinois** *show globes.*

▶ For topical use in the relief or prevention of poison ivy or poison oak dermatitis, **Parke, Davis & Co.** suggests **Ziradryl Cream and Lotion.**

▶ "The *only* motion-sickness preventive effective in a single daily dose"—**Pfizer Laboratories' Bonamine.**

▶ **Philadelphia College of Pharmacy and Science,** which incorporates Schools of Chemistry, Bacteriology and Biology, offers the undergraduate degree of B.Sc. and graduate M.Sc. and D.Sc. degrees.

▶ A "pharmaceutical adjunct" of another sort is the detail man—in this case—the representative who pre-sells *for you—in your area* the ethical pharmaceuticals of **A. H. Robins Co.**

▶ Introduced by **Schering** are **Meti-Derm Cream 0.5%** and **Meti-Derm Ointment**—"magnified potency with Meti-steroid effectiveness in allergic and inflammatory dermatoses . . . and adding dual control to Meti-steroid skin therapy—protection against infection."

▶ Two **Smith, Kline & French** insertions (1) reemphasize the sales value of the **Benzedrex** inhaler *display carton* and (2) announce in **Thora-dex Tablets** *a balanced combination of Thorazine and Dexedrine.*

▶ **Mycostatin, Squibb's** antifungal antibiotic, is now available in the form of oral tablets, vaginal tablets, and ointment.

▶ From the **Warner-Chilcott Laboratories—Sterisil Vaginal Gel.**

▶ "Top Favorites with the *Carriage* Trade" are **Winthrop Laboratories' Neo-Synephrine** *Pediatric Nasal Spray,* **pHisoHex, Pluravit Drops,** and **Pomalin.**

▶ **Wyeth** reiterates the prescription-potential inherent in **Equanil** and similarly characterizes its *new potent ataractic drug,* **Sparine.**

Look for details in the Practical Pharmacy Edition of THIS JOURNAL*—Out May 20*

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLV — *MAY 1956* — NUMBER 5 — CONSECUTIVE NO. 10

Derivatives of 3-, 4-, and 5-Phenylsalicylamides*

By LEONARD H. JULES, JOHN A. FAUST, and MELVILLE SAHYUN

General methods of synthesis and detailed examples are given for the preparation and characterization of sixty-six derivatives of 3-, 4-, and 5-phenylsalicylamide. The compounds were prepared for study as possible analgetic and/or antimicrobial agents. The analgetic potency, in the rat, of the most active compounds, N,N-dialkyl-3-phenylsalicylamides, is about four times that of salicylamide and about one-half that of codeine. N-Butyl-3-phenylsalicylamide and N-(4-hydroxyphenyl)-3-phenylsalicylamide are the most active antifungal agents of this series. The former possesses a high order of activity against *Trichophyton mentagrophytes* and *Microsporum audouini;* the latter inhibits *Candida albicans in vitro.*

ALTHOUGH many derivatives of salicylamide are reported in the literature, corresponding derivatives of 3-, 4-, and 5-phenylsalicylamide appear to have been given very little attention. As far as we are aware the only reports on these types concern certain aryl amides of 3-phenylsalicylic acid (1) and of 5-phenylsalicylic acid (2–4) which have been described as useful intermediates in the dye industry.

In view of the studies of Hart (5) and others whose reports on the analgetic properties and toxicity of salicylamide led to its use as an analgetic agent in man, it was of interest to prepare derivatives of 3-, 4-, and 5-phenylsalicylamide and study their analgetic potencies. In addition, many of the compounds in this series were also screened against the more common pathogenic fungi because certain substituted salicylamides, particularly N-aryl compounds such as salicylanilide, have found use as topical fungicides in the treatment of fungus infections.

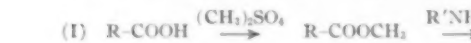
The analgetic activity of many of the compounds in this series was studied by Way, *et al.* (6). Their findings have already been reported in detail. According to these investigators, maximum analgesia was exhibited by N,N-dialkyl-3-phenylsalicylamides, such as N,N-dimethyl-3-phenylsalicylamide (Compound S.2-426), the biologic activity of which, in the rat, was about four times that of salicylamide and about half that of codeine.

Detailed results of the microbiological testing of the more active compounds of this series will be published elsewhere. Briefly, several of the N-substituted derivatives of 3-phenylsalicylamide proved to possess a high order of activity against human pathogenic fungi. Compound S.2-339, N-butyl-3-phenylsalicylamide, for example, showed fungistatic activity against *Trichophyton mentagrophytes* in a dilution of 1:512,000 in Sabouraud's agar, and in a dilution of 1:64,000 in the presence of serum. The compound also showed a high order of activity against *Microsporum audouini*, the causative agent of tinea capitis (ringworm of the scalp). The corresponding N-(4-hydroxyphenyl)-3-phenylsalicylamide, Compound S.2–203, was found to inhibit *Candida albicans in vitro.*

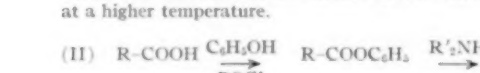
* Received July 22, 1955, from Sahyun Research Laboratories, Santa Barbara, Calif.
This investigation was supported by Cutter Laboratories, Berkeley, Calif.

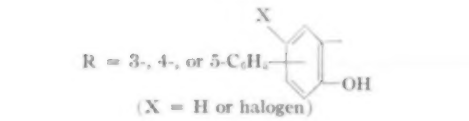
METHODS OF PREPARATION

With three exceptions which are indicated in the tables, the compounds in the present series were prepared according to one of the following methods:

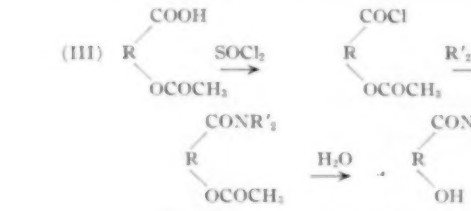


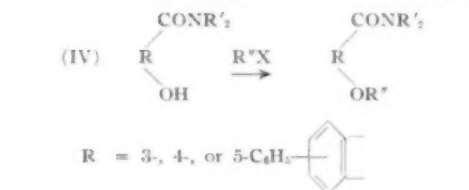
(IA). Same as method I except that the amidification was conducted in the absence of a solvent and at a higher temperature.





R′ = H or substituent as indicated in the tables.





R′ = H or substituent as indicated in the tables.
R″ = Substituent as indicated in the tables.

Most of the N-monosubstituted amides were prepared from methyl esters according to method I. Although these esters may be obtained by means of ordinary Fisher esterifications, preference was given to the more convenient and productive procedure previously described (7) involving the use of dimethyl sulfate and sodium bicarbonate in acetone.

While secondary amines did not appear to react with alkyl esters of 3-, 4-, or 5-phenylsalicylic acids, they reacted smoothly with the corresponding phenyl esters at elevated temperatures to provide good yields of N,N-disubstituted amines. The use of phenyl esters in this type of reaction has been reported previously (8).

Although somewhat less convenient than the others, method III was applicable to the preparation of either mono- or disubstituted amides, and was one of the earlier methods tried by us. In most instances it was found more advantageous to employ either method I or II.

The ethers of the 3-, 4-, or 5-phenylsalicylamide derivatives were prepared by method IV, which consists essentially of the coupling of a phenol and an alkyl halide in the presence of an alkaline agent. Alkylations with unsubstituted alkyl halides were carried out in acetone by refluxing in the presence of one molar equivalent of anhydrous potassium carbonate. Water, containing an equimolar quantity of sodium hydroxide, was employed as the reaction medium in alkylations with ethylene chlorohydrin, and it was found convenient to conduct the reaction in a closed vessel at steam bath temperature. Similar conditions were employed in alkylations with chloroacetic acid. In the preparation of the two basic ethers of this series, compounds S.2-89 and S.2-91, the appropriate dry sodium phenolate was prepared, combined with the aminoalkyl chloride in toluene, and the mixture was refluxed to effect reaction.

The properties of the intermediate esters are summarized in Table I. In Tables II, III, IV, and V are presented the pertinent data for the amides of 3-, 4-, and 5-phenylsalicylamide and derivatives thereof. The yields reported in the tables refer, in most instances, to pure products, and no attempt was made to determine the conditions leading to optimum yields. The analytical data given in the tables were obtained by R. H. McCutcheon of these Laboratories.

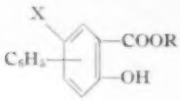
EXPERIMENTAL

The following procedures exemplify the various methods by which the compounds listed in the tables were obtained. The 3-, 4-, and 5-phenylsalicylic acids, acetyl 3-phenylsalicylic acid and the 5-halo-3-phenylsalicylic acids were obtained from the Dow Chemical Company, Midland, Michigan.

Methyl 3-phenylsalicylate.—A mixture of 21.4 Gm. (0.1 mole) of 3-phenylsalicylic acid, 15.8 Gm. (0.125 mole) of dimethyl sulfate, 10.5 Gm. (0.125 mole) of sodium bicarbonate and 100 cc. of acetone was stirred and refluxed for twenty hours. The acetone was removed by distillation, the residue was mixed with water and extracted with ether. The ether solution was washed with dilute sodium bicarbonate solution, dried over anhydrous magnesium sulfate and fractionated. Yield, 18.2 Gm., (80%); b. p. 145-148° (1 mm.); m. p. 59-60° after recrystallization from methanol.

3-Phenylsalicylamide—(Method I):—A suspension of 22.8 Gm. (0.1 mole) of methyl 3-phenylsalicylate in 200 cc. of methanol, contained in a pressure bottle, was cooled to about 10° and saturated with ammonia. The bottle was stoppered, and the mixture was allowed to remain at room temperature for six days, whereupon it became homogeneous. The tan solid residue which remained after the removal of the solvent was dissolved in isopropyl alcohol, the solution was treated with decolorizing charcoal and diluted with water. The amide separated as colorless needles. Yield, 16.2 Gm., (76%); m. p. 142-143°. The yield was increased to 90% by recovery from the mother liquor by further dilution with water.

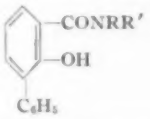
TABLE I.—INTERMEDIATE ESTERS



X	R	Position of Phenyl	M. P., °C.	Recryst. Solvent	Yield, %
H	CH_3	3	59–60[a, b]	MeOH	80
H	CH_3	4	92–93	Dil. MeOH	91
H	CH_3	5	94–95[c]	MeOH	83
H	C_6H_5	3	95–96[b]	i-PrOH	65
H	C_6H_5	4	114–115	Dil. acetone	62
H	C_6H_5	5	95–97	Acetone-MeOH	55
Br	CH_3	3	67–68[c]	Dil. i-PrOH	95
Cl	CH_3	3	77–78[d]	i-PrOH	89
I	CH_3	3	72–74	i-PrOH	71
Br	C_6H_5	3	129–131	i-PrOH	53
Cl	C_6H_5	3	110–111	Dil. acetone	38
I	C_6H_5	3	125–129	Dil. i-PrOH	34

[a] Melting point reported 54–56° (1). [b] B. p., 145–148° (1 mm.). [c] B. p., 162–167° (1 mm.). [d] B. p., 156–160° (1 mm.).

TABLE II.—DERIVATIVES OF 3-PHENYLSALICYLAMIDE



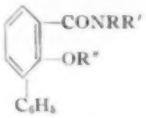
Code No.	R	R′	Method of Prepn.	Yield, %	M. P., °C.	Recryst. Solvent	Nitrogen, % Calcd.	Found
S.2-7	H	H	I	90	142–143	Dil. i-PrOH	6.57	6.37
S.2-45	H	CH_3	I	69	144–146	Dil. EtOH	6.16	6.07
S.2-16	H	C_2H_5	I	79	134–135	Dil. EtOH	5.80	5.75
S.2-46	H	$CH(CH_3)_2$	I	40	118–119	Dil. i-PrOH	5.48	5.29
S.2-62	H	CH_2CH_2OH	I[a]	86	128–129	$CHCl_3$-heptane	5.44	5.42
S.2-43	H	$CH_2CH_2C_6H_5$	III	50	99–100	Dil. i-PrOH	4.41	4.22
S.2-67	H	C_6H_5	III	34	113–115[b]	Dil. i-PrOH	4.84	4.92
S.2-66	H	C_6H_{11}[c]	III	55	182–183	$CHCl_3$-MeOH	4.74	4.72
S.2-73	H	$(CH_2)_2OCH_3$	I	52	63–64	Dil. MeOH	4.91	5.00
S.2-194	H	C_6H_4COOH(p-)	III	57	259–260 dec.	Dil. acetone	4.20	4.03
S.2-193	H	$C_6H_4NO_2$(p-)	III	44	200–201	EtOH-dil. acetone	8.38	8.16
S.2-203	H	C_6H_4OH(p-)	II[d]	68	183–184	Dil. MeOH	4.59	4.65
S.2-204	H	$C_6H_4NH_2 \cdot HCl$(p-)	[e]	60	239–241 dec.	i PrOH-ether	8.22	8.23
S.2-199	H	$C_6H_4NHCOCH_3$(p-)	III	21	279–280 dec.	AcOH	8.09	8.10
S.2-339	H	C_4H_9	I	73	71–72	Dil. MeOH	5.20	5.26
S.2-378	H	$CH_2CH(OH)CH_3$	IA	77	124–125	Dil. i-PrOH	5.16	5.21
S.2-538	H	$CH_2(CH_2)_4CH_3$	IA	52	56–57	Dil. MeOH	4.74	4.63
S.2-659	H	$CH_2CH(CH_3)_2$	II	71	77–79	Dil. MeOH	5.20	5.14
S.2-426	CH_3	CH_3	II	65	95–96[f]	Dil. MeOH	5.80	5.69
S.2-51	C_2H_5	C_2H_5	II	48	91–92	MeOH	5.20	5.12
S.2-56	C_5H_{10}[g]		III	38	111–112	i-PrOH	4.98	4.91
S.2-455	C_4H_8[h]		II	73	80–81	MeOH	5.35	5.23

[a] Also prepared by method IA. [b] Melting point reported, 120–121° (1). [c] C_6H_{11} = cyclohexyl. [d] Also prepared by method III. [e] Prepared by the catalytic reduction of S.2-193 in ethanol using 5% palladium on charcoal as the catalyst. [f] B. p., 188–190° (2–3 mm.). [g] C_5H_{10} = pentamethylene. [h] C_4H_8 = tetramethylene.

N-(2-Hydroxypropyl)-3-phenylsalicylamide—(Method IA):—A mixture of 37 Gm. (0.16 mole) of methyl 3-phenylsalicylate and 24 Gm. (0.32 mole) of 1-amino-2-propanol was refluxed for six hours, cooled and poured into 300 cc. of cold dilute hydrochloric acid. The oil which separated solidified, and the solid was recrystallized from dilute isopropyl alcohol. Yield, 33.5 Gm., (77%); m. p. 124-125°.

Phenyl-3-phenylsalicylate.—An intimate mixture of 128.5 Gm. (0.6 mole) of 3-phenylsalicylic acid, 56.5 Gm. (0.6 mole) of phenol and 30.7 Gm. (0.2 mole) of phosphorous oxychloride was heated at 105-110°, cautiously at first, until the evolution of gas had practically ceased (2–3 hours). The red liquid reaction mixture was poured while hot into a stirred solution of 85 Gm. of sodium carbonate decahydrate in 300 cc. of water at room temperature. The oil solidified and the mixture was heated at 90° for about ten minutes and filtered. After the solid had been thoroughly washed with hot water, it was

TABLE III.—DERIVATIVES OF 3-PHENYLSALICYLAMIDE



Code No.	R	R′	R″	Method of Prepn.	Yield %	M. P., °C.	Recryst. Solvent	Nitrogen, % Calcd.	Found
S.2-39	H	H	CH_3	IV	73	177–178	i-PrOH	6.16	6.14
S.2-174	H	H	CH_2COOH	IV	22	196–197	i-PrOH	5.16	4.98
S.2-47	H	H	CH_2CH_2OH	IV	53	138–140	$CHCl_3$-heptane	5.44	5.30
S.2-91	H	H	$CH_2CH_2N(C_2H_5)_2$	IV	81	82–84	MeOH	8.71	8.81
S.2-396	H	H	$CH_2CH{=}CH_2$	IV	74	121–122	Dil. i-PrOH	5.53	5.45
S.2-319	H	CH_3	CH_2COOH	IV	47	187–188	Dil. EtOH	4.98	4.91
S.2-97	H	CH_3	CH_3	I	79	127–128	i-PrOH	5.81	5.87
S.2-446	CH_3	CH_3	CH_3	IV	62	76–77	Dil. i-PrOH	5.49	5.32
S.2-447	CH_3	CH_3	$CH_2CH{=}CH_2$	IV	56	Oil[a]	.....	4.98	4.95
S.2-448	CH_3	CH_3	CH_2CH_2OH	IV	74	Oil[b]	.....	4.90	4.83
S.2-459	CH_3	CH_3	C_2H_5	IV	68	75–77[c]	Pet. ether	5.20	5.12
S.2-460	CH_3	CH_3	$COCH_3$	[e]	53	99–102	Dil. EtOH	4.94	4.96
S.2-461	C_2H_5	C_2H_5	$CH_2CH{=}CH_2$	IV	51	Oil[d]	.....	4.52	4.48

[a] B. p., 180–183° (1 mm.). [b] B. p., 194–212° (1 mm.). [c] B. p., 170–174° (4–5 mm.). [d] B. p., 183–187° (4 mm.).
[e] Prepared by the acetylation of compound S.2-426 with acetyl chloride in pyridine.

TABLE IV.—DERIVATIVES OF 4- AND 5-PHENYLSALICYLAMIDE

Code No.	R	R′	R″	Method of Prepn.	Yield, %	M. P., °C.	Recryst. Solvent	Nitrogen, % Calcd.	Found
4-Phenyl									
S.2-240	H	H	H	I	88	224–226	EtOH	6.57	6.54
S.2-241	H	CH_3	H	I	90	217–218	EtOH	6.16	6.08
S.2-346	H	H	CH_2CH_2OH	IV	35	109–111	$CHCl_3$-heptane	5.44	5.11
S.2-251	H	CH_3	CH_2CH_2OH	IV	62	85–86	Dil. i-PrOH	5.16	5.06
S.2-452	CH_3	CH_3	H	II	86	165–166	i-PrOH	5.80	5.82
S.2-563	H	C_4H_9	H	II	95	129–130	Dil. MeOH	5.20	5.12
5-Phenyl									
S.2-254	H	H	H	I	89	177–178	Dil. acetone	6.57	6.56
S.2-255	H	CH_3	H	I	78	183–184	Dil. acetone	6.16	6.12
S.2-256	H	CH_3	CH_2CH_2OH	IV	52	130–131	$CHCl_3$-EtOH	5.16	5.09
S.2-345	H	H	CH_2CH_2OH	IV	54	187–189	MeOH	5.44	5.36
S.2-399	H	CH_3	$CH_2CH{=}CH_2$	IV	96	113–114	Dil. i-PrOH	5.24	5.37
S.2-450	CH_3	CH_3	H	II	69	171–172	Dil. i-PrOH	5.80	5.81
S.2-564	H	C_4H_9	H	II	84	86–87	Dil. MeOH	5.20	5.08

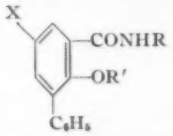
recrystallized from isopropyl alcohol. Yield, 113 Gm., (65%); m. p. 95-96°[1].

N - (3 - Phenylsalicyloyl)pyrrolidine—(Method II).—Pyrrolidine, 21.3 Gm. (0.3 mole) was combined with 58.1 Gm. (0.2 mole) of phenyl 3-phenylsalicylate and the mixture was heated under reflux for four hours. After the excess amine and most of the phenol had been removed by vacuum distillation, the mixture was steam distilled to remove the remainder of the phenol. The residual oil was washed with water by decantation, dissolved in 300 cc. of methanol and the solution was treated with decolorizing carbon. Dilution of the solution with water promoted crystallization of the product. After a subsequent recrystallization of this material from methanol there was obtained 39 Gm. (73%) of yellowish platelets, m. p., 80-81°.

[1] Reported m. p., 90–92° (1).

N - Cyclohexyl - 3 - phenylsalicylamide—(Method III).—A mixture of 51.2 Gm. (0.2 mole) of acetyl 3-phenylsalicylic acid, 42 cc. (0.6 mole) of thionyl chloride and 50 cc. of benzene was refluxed for two hours, and distilled under reduced pressure to an oil. Fresh portions of benzene were distilled from the oil in order to remove the last traces of thionyl chloride, and the oily acid chloride was dissolved in 100 cc. of dioxane. Thirty-two grams of this solution (containing 0.04 mole of acid chloride) was added dropwise to a cold, stirred solution of 9.9 Gm. (0.1 mole) of cyclohexylamine in 25 cc. of dioxane. After the mixture had been stirred at room temperature for one hour, it was chilled, diluted with water, and acidified with hydrochloric acid. The oil which separated was dissolved in ethanol, 40 cc. of 20% sodium hydroxide was added and the mixture was refluxed for three hours, the last 15 minutes of which was in the presence of charcoal. The mixture was

TABLE V.—5-HALO-3-PHENYLSALICYLAMIDES



Code No.	R	R′	X	Method of Prepn.	Yield, %	M. P., °C.	Recryst. Solvent	Nitrogen, % Calcd.	Found
S.2-41	H	H	Br	I	66	202–203	Dil. EtOH	4.80	4.64
S.2-100	H	H	Cl	I	70	191–192	Dil. i-PrOH	5.65	5.59
S.2-118	H	H	I	I	74	195–196	Dil. i-PrOH	4.13	3.99
S.2-70	CH_3	H	Br	I	86	189–190	Dil. i-PrOH	4.58	4.75
S.2-238	CH_3	H	I	I	80	200–201	i-PrOH	3.96	3.85
S.2-71	$CH(CH_3)_2$	H	Br	I	81	134–135	dil. i-PrOH	4.19	4.18
S.2-76	CH_2CH_2OH	H	Br	IA	75	181–182	EtOH-$CHCl_3$-heptane	4.17	4.14
S.2-102	CH_2CH_2OH	H	Cl	IA	98	186–187	Dil. MeOH	4.80	4.68
S.2-119	CH_2CH_2OH	H	I	IA	93	177–178	Dil. MeOH	3.66	3.48
S.2-539	C_4H_9	H	Br	II	46	76–77	Heptane	4.02	4.01
S.2-749	C_4H_9	H	Cl	II	54	58–60	Dil. i-PrOH	4.61	4.46
S.2-801	C_4H_9	H	I	[a]	61	93–94	Dil. EtOH	3.54	3.45
S.2-642	OH	H	Cl	I	68	165 dec.	Dil. MeOH	5.31	5.00
S.2-645	$NH_2.HCl$	H	Cl	I	28	225–227	EtOH-Et_2O		[b]
S.2-58	H	CH_2CH_2OH	Br	IV	37	146–147	$CHCl_3$-heptane	4.17	4.24
S.2-89	H	$CH_2CH_2N(i\text{-}C_3H_7)_2$	Br	IV	28	123–125	Dil. MeOH	6.68	6.57
S.2-78	CH_3	CH_3	Br	IV	80	115–116	Dil. i-PrOH	4.37	4.46
S.2-82	CH_2CH_2OH	CH_2CH_2OH	Br	IV	53	162–163	$CHCl_3$-heptane	3.68	3.76

[a] Prepared by the iodination of compound S.2-339 with ICl in acetic acid. [b] Chloride analysis: Calcd. 11.85; found 11.63.

filtered and acidified to precipitate 9 Gm. of a yellow solid which, after recrystallization from a mixture of chloroform and methanol, weighed 6.7 Gm. (55%); m. p. 182-183°.

2-Allyloxy-3-phenylbenzamide—(Method IV).—A stirred mixture of 42.6 Gm. (0.2 mole) of 3-phenylsalicylamide, 24.2 Gm. (0.2 mole) of allyl bromide, 27.6 Gm. (0.2 mole) of anhydrous potassium carbonate and 200 cc. of acetone was refluxed for eight hours and poured into approximately 700 cc. of water. The mixture was made strongly alkaline with sodium hydroxide and the solid was isolated. Recrystallization of the solid from a mixture of isopropyl alcohol and water (2:1) yielded 37.5 Gm. (74%) of white needles; m. p., 121-122°.

When ethylene chlorohydrin was used as the alkylating agent the procedure was modified as follows: equimolar quantities of the phenol, ethylene chlorohydrin and sodium hydroxide were combined in water (150 cc. per 0.1 mole), and the mixture, contained in a pressure bottle, was heated on a steam bath for six hours. The product which separated from the cooled reaction mixture was collected on a filter and purified by recrystallization from an appropriate solvent. In one instance, the product separated as an oil from the reaction mixture, and was extracted with chloroform and purified by distillation.

Compounds S.2-174 and S.2-319 were also prepared according to this modification except that approximately two molar equivalents of sodium hydroxide were used, and the crude products were obtained by acidification of the cooled reaction mixture.

For the synthesis of aminoalkyl ethers (Compounds S.2-89 and S.2-91), the dry sodium phenolate prepared from the phenol and sodium ethylate in alcohol, was combined with an equimolar amount of the aminoalkyl chloride in toluene, and the mixture was stirred and refluxed for five hours. The cooled toluene solution was washed with water and extracted with dilute hydrochloric acid. The acidic aqueous extract was made alkaline with sodium hydroxide to precipitate the basic ether base which was purified by crystallization.

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Studies on the Toxicity of Acetoglycerides*

By ANTHONY M. AMBROSE and DOROTHY J. ROBBINS

Results of studies are presented on the toxicity of two "acetoglycerides" (acetostearin and aceto-olein), administered by various routes to albino rats, rabbits, and guinea pigs. No toxic effects were observed in rats receiving single oral doses of 4 Gm./Kg. nor in rats consuming diets containing 5, 10, or 20 per cent of the acetostearin for fourteen weeks, nor in rats on diets containing up to 4 per cent acetostearin or 1 per cent aceto-olein for fifty-seven weeks. No blood changes or major histopathological visceral damage were observed in rats used for the subacute and chronic toxicity experiments. Hypoplasia of the testes and suppression of spermatogenesis, which were observed with the acetoglycerides, are considered to be due to other factors. Acetostearin administered intravenously, in rabbits, in doses of 80 to 100 mg./Kg. daily for fifteen days was nontoxic, had questionable effect on clotting time, and was completely removed from circulating blood within thirty minutes. Daily inunction of guinea pigs with 4 ml. of a 30 per cent emulsion for forty-five days produced no local or systemic reactions. No sensitization or allergic responses were observed in guinea pigs.

THE ACETYLATION of saturated or unsaturated monoglycerides, prepared from animal fats and vegetable oils consisting primarily of stearic acid (acetostearin) and oleic acid (aceto-olein), has resulted in the formation of new-type fats of unusual properties (1–3). These modified fats, referred to collectively as "acetoglycerides," are of potential value in the preparation of pharmaceuticals and cosmetics (4) and as edible fats (5). Studies on the absorption and digestibility of various acetoglycerides have been presented in another publication (6).

In cooperation with the Southern Utilization Research Branch we have made a study on the acute, subacute, and chronic oral toxicities of these acetoglycerides.

EXPERIMENTAL PROCEDURES AND RESULTS

In the present studies two synthetic acetoglycerides have been investigated, namely, diacetomonostearin and diacetomono-olein. The acetostearin was prepared by acetylation of molecularly distilled monostearin and the aceto-olein was prepared from purified oleic acid by interesterification with glycerol prior to acetylation. Both were prepared and supplied by the Southern Utilization Research Branch.

Oral Administration–Single Dose by Stomach Tube–Rats.—The acute toxicity of a mixture of acetostearin and aceto-olein was determined in mature male and female rats by gastric intubation. For this purpose a 40% emulsion was prepared as follows: 20 Gm. of acetostearin and 20 Gm. of aceto-olein were emulsified with 2 Gm. of Span® 20[1] and 1 Gm. of Tween® 60[1]. Since the completion of these studies, we have found that a suitable emulsion can be prepared with glycerol-monostearate (Tegin®)[1]. A 20% emulsion of either acetostearin or aceto-olein was prepared with 2% Tegin®.

In twenty rats, ten of each sex, receiving single oral doses of 4 Gm./Kg., no obvious toxic symptoms or ill effects were observed within the first twenty-four hours following administration or during the next four weeks.

Intravenous Administration–Repeated Doses–Rabbits.—Studies were made in rabbits by daily intravenous administration of a 10% emulsion of acetostearin on clotting time and on rate of disappearance, as determined by turbimetric measurements, from circulating blood.

In rabbits, daily intravenous injections of 80 to 100 mg. of acetostearin for fifteen days were nontoxic. Histological examination of the visceral organs showed no pathological changes due to the acetoglycerides. On clotting time only a slight and transitory effect was observed. Disappearance of acetostearin from the blood, as determined turbimetrically, showed that the administered acetostearin, 100 mg./Kg., was completely removed from circulating blood (plasma) within fifteen to thirty minutes.

Skin Irritation and Allergic Responses–Guinea Pigs.—The effect of acetoglycerides upon the skin was determined on albino guinea pigs after daily cutaneous application of an emulsion of acetoglycerides, consisting of 20% acetostearin, 10% aceto-olein, 2% Span® 20, and 1% Tween® 60. The animals were prepared by removing superfluous hair from the flanks and ventral surface with clippers.

The daily inunction of guinea pigs, involving approximately one-third of the total body surface, with 4 ml. of the 30% emulsion for forty-five days resulted in no local irritation or systemic reactions. No sensitization or allergic response was observed in guinea pigs receiving the acetoglycerides by cutaneous application.

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We also wish to acknowledge the technical assistance of Miss Iva V. Streeter and Mr. M. Nonaka of the Poultry Meat Unit of this Branch for determining the peroxide values and vitamin E values, respectively.

[1] Brand of emulsifiers: Span and Tween supplied by Atlas Powder Company, and Tegin supplied by Goldschmidt Chemical Corporation. The use of proprietary names does not imply endorsement of these products.

Oral Administration in the Diet for 14 Weeks–Rats.—As a preliminary study to more extensive studies, five weanling female albino rats were placed on each of four diets for fourteen weeks, the composition of which is given in Table I. One group of rats was placed on the basal diet, and the remaining groups were placed on diets containing 5, 10 and 20% acetostearin. All rats had free access to their respective diets and water. Body weights and food consumption were recorded weekly.

This experiment was terminated at fourteen weeks, when all rats were autopsied. The differences in growth between the controls and experimental animals were not significant. Acetostearin consumption for rats on the 20% dietary intake varied from approximately 23 Gm./Kg./day during the first week to approximately 10 Gm./Kg./day at fourteen weeks. For rats on 5 and 10% acetostearin, the daily dietary intake/Kg. was proportionately lower. At necropsy, no gross postmortem changes were observed.

TABLE I.—COMPOSITION OF DIETS

Constituents	Basal	Experimental		
	%	%	%	%
Acetostearin	0	5	10	20
Corn meal (yellow degerminated)	73	68	63	51
Casein (ground)	10	10	11	12
Linseed oil cake meal	10	10	10	10
Alfalfa meal (ground)	2	2	2	2
Bone ash	1.5	1.5	1.5	1.5
Sodium chloride	0.5	0.5	0.5	0.5
Cod liver oil (U. S. P.)	3	3	3	3

Oral Administration in the Diet for 57 Weeks–Rats.—In these studies, two experiments were conducted in which groups of weanling male albino rats were used. In one experiment six groups of five rats each were fed on diets containing 0, 0.25, 0.5, 1, 2, and 4% acetostearin. In the second experiment four groups of five rats each were fed on diets containing 0, 0.25, 0.5, and 1% aceto-olein. The basal diet to which the acetoglycerides were added was the same as that used in the previous experiments, Table I. The vitamin E content of the basal diet was equivalent to approximately 20 μg./Gm. of diet and for the acetostearin approximately 68 μg./Gm. The peroxide values for the basal diet and for the diet containing 10% acetostearin after standing for three weeks at 25° were approximately 0.002 and 0.0026 meq./Gm. of diet respectively. These values are in close agreement with the peroxide values of the freshly prepared diets which for all practical purposes are negligible. All diets were fed *ad libitum* and free access to water was permitted at all times.

In Table II, data are summarized on the final body weights and organ weights of rats on the various dietary levels of acetostearin and aceto-olein for fifty-seven weeks (four hundred days). Growth of rats on the experimental diets was not significantly different from that of the controls. A few deaths occurred in different groups and these were not related to treatment and were therefore not significant.

Organ weights, consisting of liver, kidneys, testes, spleen, heart, and adrenals, were determined routinely on all rats. With the exception of the adrenals, the average organ weights, together with the standard error of the mean, are summarized in Table II. Except for the testes all organ weights compare favorably with those of the controls. Hypoplasia of the testes was observed with rats on the 0.25 and 0.5% acetostearin and on 0.25, 0.5, and 1% aceto-olein. The testes of rats on acetoglyceride diets were much smaller both in terms of absolute weight and as related to body weight than were those of the control rats. The significance of this observation will be discussed below in conjunction with histopathological studies and in more detail in a subsequent publication.

Histopathological studies on hematoxylin-eosin stained sections were made on all of the organs weighed and, in addition, on the thyroid, intestine, stomach, pancreas, and bladder. Except for the testes no outstanding gross or microscopic pathological differences were observed between the tissues of the experimental groups and those of the control groups. Testicular hypoplasia and suppression of spermatogenesis of varying degrees were observed in all rats. In the controls one out of nine rats showed severe suppression of spermatogenesis. With the acetostearins, mild to moderate suppression of spermatogenesis was observed in all rats on the 4% diet; in three on the 2% diet; none on the 1% diet; in two on the 0.5% diet, and in four on the 0.25% diet. With the aceto-oleins all rats showed variable degrees of suppression of spermatogenesis. The significance of the hypoplasia of the

TABLE II.—AVERAGE ORGAN WEIGHTS OF MALE RATS RECEIVING ACETOSTEARIN OR ACETO-OLEIN IN THEIR DIETS FOR 400 DAYS

Supplements Fed				Organ Wt. (Gm./100 Gm. Body Wt.) ± S. E.				
Name	Concn. (Wt. %)	No. of Rats	Body Wt. Gm.	Liver	Kidneys	Testes	Spleen	Heart
Acetostearin	0.00	4	383 ± 16.4	3.13 ± 0.13	0.59 ± 0.04	0.87 ± 0.08	0.17 ± 0.01	0.31 ± 0.02
	0.25	5	384 ± 14.1	3.57 ± 0.14	0.62 ± 0.02	0.58 ± 0.07[a]	0.17 ± 0.08	0.32 ± 0.02
	0.50	5	388 ± 10.9	2.96 ± 0.05	0.63 ± 0.03	0.57 ± 0.05[a]	0.17 ± 0.01	0.35 ± 0.02
	1.00	5	390 ± 10.5	3.06 ± 0.08	0.59 ± 0.02	0.72 ± 0.03	0.16 ± 0.01	0.30 ± 0.01
	2.00	5	388 ± 15.7	3.18 ± 0.11	0.63 ± 0.03	0.73 ± 0.06	0.16 ± 0.01	0.29 ± 0.02
	4.00	4	386 ± 5.7	3.22 ± 0.18	0.62 ± 0.03	0.62 ± 0.09	0.20 ± 0.04	0.33 ± 0.05
Aceto-olein	0.00	5	358 ± 6.1	3.03 ± 0.15	0.62 ± 0.01	0.77 ± 0.09	0.17 ± 0.01	0.31 ± 0.01
	0.25	4	378 ± 5.3	2.95 ± 0.15	0.63 ± 0.02	0.63 ± 0.04[a]	0.18 ± 0.01	0.32 ± 0.01
	0.50	4	371 ± 17.4	3.20 ± 0.11	0.66 ± 0.06	0.60 ± 0.02[a]	0.17 ± 0.00	0.29 ± 0.01
	1.00	5	385 ± 8.4	3.17 ± 0.11	0.62 ± 0.07	0.55 ± 0.04[a]	0.18 ± 0.01	0.31 ± 0.02
Controls (Comb.)	0.00	9	369 ± 8.5	3.06 ± 0.11	0.61 ± 0.02	0.82 ± 0.06	0.17 ± 0.05	0.31 ± 0.01

[a] P = <0.01.

testes and the suppression of spermatogenesis with the acetoglycerides is not understood at this time, since it has also been observed in other studies (unpublished data) with materials totally unrelated to the acetoglycerides (feather-meal protein and allethrin). However, preliminary observations, in another series of studies with acetoglycerides, would suggest that the testicular hypoplasia and suppression of spermatogenesis was possibly due to an insufficient amount of vitamin E in the diet in the presence of stress (acetoglycerides) rather than to other causes (peroxide formation).

Hematological studies made at approximately four-month intervals showed no toxic effect on the blood at any of the dosages of the acetoglycerides.

SUMMARY

1. When acetoglycerides were administered to rats by gastric intubation in massive doses of 4 Gm./Kg., there was no evidence of any toxicity.

2. Intravenously, in rabbits, daily doses of 80 to 100 mg./Kg. of a 10% emulsion of acetostearin for fifteen days was nontoxic, had questionable effects on clotting time, and was completely removed from circulating blood within 30 minutes. Histological examination of the visceral organs showed no pathological changes.

3. In guinea pigs, daily inunctions for forty-five days with 4 ml. of a 30% emulsion of acetoglycerides produced no local irritation or systemic reactions. No sensitization or allergic response was observed in albino guinea pigs receiving the acetoglycerides by cutaneous application.

4. When acetostearin was fed to rats in concentrations of 5, 10, and 20% of the diet for fourteen weeks, there was no evidence of toxicity as determined by growth, food intake, and mortality. Rats maintained for fifty-seven weeks on diets containing acetostearin in concentrations of 4% or less of the diet and aceto-olein in concentrations of 1% or less of the diet exhibited no appreciable ill effects as ascertained by growth, food intake, mortality, periodic hematological examinations, and histopathological examination of visceral organs. However, hypoplasia of the testes and suppression of spermatogenesis, which were observed with the acetoglycerides, is thought to be due to other factors, since this phenomenon was also observed by us with other materials, such as feathermeal protein, to some extent with allethrin, and in vitamin E deficiencies (unpublished data).

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An Investigation of Triterpenes as Steroid Hormones*

By DUANE G. WENZEL† and GILBERT H. EMICK, JR.

Five triterpenes were screened for desoxycorticosterone, cortisone, estrogenic, and androgenic activity. None of the compounds was active in any of the tests.

THIS STUDY was initiated in part on the basis of evidence demonstrating the effect of the triterpene glycyrrhetinic acid on electrolyte balance and its use in the treatment of Addison's disease (1–10). Further reason for the study was obtained by one of the investigators (Wenzel) during the course of a chemical examination of the fruit of the Osage orange (11). Dairy farmers consistently reported that their cows "went dry" after eating this fruit, thus suggesting the possibility of some hormonal action. The facts that the isomeric triterpenes, lupeol and lurenol, are contained in the fruit of the Osage orange and that the triterpenes have a structural similarity to the steroid hormones made the present study advisable.

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EXPERIMENTAL

Materials.[1]—Glycyrrhetinic acid was prepared in part by the method of Ruzicka (12) from extract of glycyrrhiza and was also obtained from Mac Andrews and Forbes Company, as was the am-

[1] All melting points obtained are uncorrected.

monium glycyrrhizinate, through the courtesy of C. K. Swift. The acetate of glycyrrhetinic acid and the following triterpenes were prepared according to usual acetylation procedures. Glycyrrhetinic acid acetate melted at 314–316°. The reported m. p. is 316° (12).

Lupeol and lurenol were obtained as a mixture from the dried Osage orange according to the method of Swift (13) and separated as the isomers as described by Wagner and Harris (14). The acetates of lupeol and lurenol melted at 220° and 135°, respectively. Those reported are 222.3–222.8° and 132.5–133.5° (14).

Oleanolic acid was isolated from cloves by the method of Picard, *et al.* (15). The acetate melted at 268°; reported 268° (16).

Ursolic acid was prepared according to Brieskorn (17) from the crude product obtained from A. C. Garland of the National Cranberry Association. The acetate melted at 285–289°; reported 289–290° (18).

Rat Cold Survival Test.—A modification of the procedure of Selye and Schenker for testing adrenal cortical extracts was used (19). Complete litters of Sprague Dawley strain rats twenty-four±one days old and weighing 37–55 Gm. were used. The animals were bilaterally adrenalectomized under 25 mg./Kg. sodium pentobarbital anesthesia. Occasionally supplemental etherization was also necessary. The rats were divided into 14 groups of 10 or 11 animals each with litter mates evenly distributed and the group mean weights and sex ratios kept as equal as possible. Postoperative deaths reduced the size of some groups. After the adrenalectomies each group was subcutaneously administered one of the following per animal: sesame oil, 0.2 ml.; desoxycorticosterone acetate, 1 mg.; ammonium glycyrrhizinate and the acetates of glycyrrhetinic acid, lupeol, lurenol, oleanolic acid and ursolic acid, 5 mg. Test drugs were injected in sesame oil in such concentration as to provide the required dose in a volume of 0.2 ml. The triterpenes were also administered perorally to separate groups in the same dosage as 1% suspensions with acacia as the suspending agent.

On the day following the adrenalectomy two more doses were given at 8:00 a. m. and 4:00 p. m. The fourth and last dose was administered at 4:00 a. m. on the second day after surgery. At 7:00 a. m. the rats were placed in a rack of cages and transferred to a refrigerator maintained at a temperature of 5–7°. Rapid inspections of the animals were made at half-hour intervals. The criterion of the test was the time elapsed from placing the animals in the refrigerator until death as determined by loss of the corneal reflex.

As indicated in Table I, the triterpenes were ineffective in prolonging the cold survival time of adrenalectomized rats. Since the completion of this work similar negative results have been reported by others for licorice extract and glycyrrhetinic acid (8).

Glycogenic Activity Test.—The relationship between the adrenal cortical hormones and liver glycogen deposition was first observed by Britton and Silvette (20). It was later demonstrated that liver glycogen is rapidly depleted in an adrenalectomized rat and the administration of corticosterone, cortisone or related compounds with an oxygen at C-11 would cause an increase in liver glycogen deposition (21). A modification of the procedure of Venning (22) was used to determine the glycogenic activity.

Twelve Sprague Dawley rats per group were used for each of the triterpenes previously indicated, cortisone and a control. Animals were distributed with regard to sex and littermates as in the rat cold survival test. At the age of forty±one days the rats were given drinking water containing 0.9% sodium chloride and 5% glucose *ad libitum* to combat postoperative shock. Food consisted of Purina Laboratory Chow which compares favorably in composition to the McCollum lactation diet as used by Venning (22). The adrenals were removed as in the rat cold survival test. On the first postoperative day animals were allowed free access to food and 0.9% sodium chloride solution. Food was removed at 5:00 p. m. on the second postoperative day but the saline continued. On the third day following adrenalectomy the saline was removed and one hour later a series of seven injections of the test drugs, cortisone and a control, at forty-five minute intervals was begun. All injections were made via the intraperitoneal route as acacia suspensions. The triterpenes were administered in single doses of 40 mg./Kg. or a total of 280 mg./Kg. Cortisone was given in 1 mg./Kg. doses or a total of 7 mg./Kg., while the control group received an equivalent volume of an acacia suspension. One hour after the last injection, each rat was killed by a blow on the head, the liver quickly removed and plunged into a test tube containing 4 ml. of hot 30% potassium hydroxide. Glycogen content of the livers was determined by the method of Good (23).

TABLE I.—EFFECT OF TRITERPENES ON COLD SURVIVAL TIME OF ADRENALECTOMIZED RATS

Drug[a]	Mean Survival Time Time, Min. ± S. E.[b] Subcutaneous	Oral
Control (sesame oil)	274 ± 23.7	....
Desoxycorticosterone	433 ± 15.1	....
Glycyrrhetinic acid	306 ± 12.8	320 ± 15.7
NH_4 glycyrrhizinate	303 ± 29.3	338 ± 17.7
Lupeol	324 ± 10.8	279 ± 22.8
Lurenol	306 ± 15.4	273 ± 18.1
Oleanolic acid	233 ± 12.0	267 ± 22.0
Ursolic acid	264 ± 43.7	300 ± 34.2

[a] Acetates with the exception of ammonium glycyrrhizinate. Total dose of triterpenes 20 mg., of desoxycorticosterone 4 mg.

[b] S. E. = $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$

The results are expressed in Table II as milligrams of glucose per one hundred grams of rat body weight. This procedure eliminates the need for weighing the excised livers. The average rat weight in this experiment was approximately 100 Gm., therefore the amount of glucose reported is that found in the liver of the average rat. From the values indicated it is apparent that the triterpenes tested are inactive as cortisone substitutes when evaluated by the liver glycogen test. It is believed that if any activity were present it should have been detectable with

TABLE II.—EFFECT OF TRITERPENES ON LIVER-GLYCOGEN DEPOSITION OF ADRENALECTOMIZED RATS

Drug[a]	Glucose, mg. per 100 Gm Rat Wt. ± S. E.[b]
Control (acacia suspension)	5.3 ± 0.48
Cortisone	45.4 ± 3.06
Glycyrrhetinic acid	3.6 ± 0.63
Ammonium glycyrrhizinate	5.2 ± 1.23
Lupeol	2.4 ± 0.68
Lurenol	3.9 ± 0.43
Oleanolic acid	4.6 ± 0.66
Ursolic acid	3.2 ± 0.51

[a] Acetates with the exception of ammonium glycyrrhizinate. Total dose of triterpenes 280 mg./Kg., of cortisone 7 mg./Kg.

[b] S. E. = $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$

TABLE III.—EFFECT OF TRITERPENES ON RAT UTERINE WEIGHT AND LENGTH

Drug[a]	Mean Uterine Wt., mg. ± S. E.[b]	Mean Uterine Length, mm. ± S. E.[b]
Control (sesame oil)	24.4 ± 1.41	42.0 ± 1.39
Estrone	116.0 ± 5.68	55.5 ± 2.41
Glycyrrhetinic acid	30.1 ± 1.49	53.6 ± 1.99
NH_4 glycyrrhizinate	31.1 ± 1.53	51.0 ± 0.96
Lupeol	24.4 ± 1.88	47.8 ± 1.41
Lurenol	26.3 ± 1.41	47.4 ± 1.82
Oleanolic acid	28.0 ± 0.84	51.9 ± 1.64
Ursolic acid	28.0 ± 1.26	52.0 ± 1.62

[a] Acetates with the exception of ammonium glycyrrhizinate. Total dose of triterpenes 12 mg., of estrone 0.012 mg.

[b] S. E. = $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$

TABLE IV.—EFFECT OF TRITERPENES ON IMMATURE CASTRATED RAT PROSTATE AND SEMINAL VESICLES

Drug[a]	Prostate and Seminal Vesicles Mean Wt., mg. ± S. E.[b]	Seminal Vesicles Mean Wt., mg. ± S. E.[b]
Control (sesame oil)	46.2 ± 1.58	16.2 ± 0.53
Testosterone	228.0 ± 5.16	68.9 ± 1.94
Glycyrrhetinic acid	42.0 ± 4.36	15.5 ± 0.32
NH_4 glycyrrhizinate	42.5 ± 1.73	16.1 ± 0.64
Lupeol	41.2 ± 1.26	16.1 ± 0.61
Lurenol	42.4 ± 1.49	17.1 ± 0.81
Oleanolic acid	40.5 ± 1.50	15.2 ± 0.52
Ursolic acid	41.4 ± 1.28	16.4 ± 0.46

[a] Acetates with the exception of ammonium glycyrrhizinate and testosterone which was the propionate. Total dose of triterpenes 20 mg., of testosterone propionate 10 mg.

[b] S. E. = $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$

the fortyfold difference in the doses of cortisone and the triterpenes.

Estrogenic Activity Test.—The general method of Lauson, *et al.* (24) was used to determine estrogenic activity. The triterpenes previously described, estrone as a standard and cottonseed oil as a control were tested on separate groups of female Sprague Dawley rats. Sixteen litters of four females per litter were divided into groups of eight with each of the littermates in separate groups. All rats were twenty-two±one days old when the assay was begun.

The triterpenes were injected subcutaneously in doses of 2 mg. in 0.2 ml. of cottonseed oil twice daily for three days. Estrone was similarly administered in doses of 2 μg. in the same volume of oil while the control was given the oil alone. On the fourth day, seventy-two to seventy-five hours after the first injection the rats were sacrificed and the uteri removed, weighed and measured.

As shown in Table III the triterpenes, although administered in 1,000-fold greater dosage than estrone, failed to cause any apparent increase in the mean uterine weight or length.

Androgenic Activity Test.—The procedure used was patterned after that of Burn (25). Groups of 8 Sprague Dawley rats twenty-two±one days old were used. The groups were obtained from 16 litters of four males per litter and were set up as in the estrogen test.

The animals were castrated and fifteen days later subcutaneous administration of the six triterpenes, testosterone propionate and a sesame oil control was started. Doses were 4 mg. of the triterpenes and 2 mg. of testosterone propionate in 0.5 ml. of sesame oil. This amount of oil was also given as a control. Injections were made daily for five days. On the sixth day the prostate and seminal vesicles were removed as a unit and placed in Bouin's solution for two to three days. The glands were then pressed between blotting paper and weighed together and as the separate seminal vesicle.

A comparison of the results in Table IV indicates no androgenic activity of the triterpenes tested.

DISCUSSION

The results, although entirely negative, corroborate the findings of others that glycyrrhetinic acid does not possess desoxycorticosterone activity when measured by the cold stress test (26). While it cannot be said that the triterpenes do not have cortisone activity solely on the basis of the negative glycogen deposition test, there has been no suggestion of such activity in the literature. The negative estrogenic and androgenic tests may be considered as reasonably conclusive evidence for the lack of these types of activity.

SUMMARY

1. Five triterpenes: glycyrrhetinic acid, lupeol, lurenol, oleanolic acid, and ursolic acid were prepared as the acetates. Glycyrrhetinic acid was also obtained as the ammonium salt.

2. These triterpenes were tested for desoxycorticosterone activity by the cold stress test in adrenalectomized rats; for cortisone activity by the deposition of glycogen in adrenalectomized rats; for estrogenic activity by uterine weight gain in immature female rats; and for androgenic activity by the effect on the prostate and seminal vesicles of immature castrated rats.

3. There was no indication of hormonal activity of the triterpenes with any of the tests performed.

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Ion Exchange Resin Dye Compounds for Use in Indirect Determination of Gastric Acidity*

By GEORGE S. SHARP, S. SHANKMAN, and MERWIN S. KENDIS

The results of a systematic *in vitro* investigation of the ion exchange resin dye compounds are reported. The indicators were selected for their readily recognizable colors. Representative combinations of resins and indicators were prepared and evaluated. A typical procedure for 1 of 21 compounds prepared is presented. An improved *in vitro* evaluation technique is outlined, and *in vitro* elution studies made of 8 classical types of resin indicator compounds are presented. The compound type most nearly conforming to stated requirements consisted of a weak cation exchange resin combined with a strong base indicator. Carboxylic cation exchange resins are suitable for use in tubeless tests for gastric acidity. No suitable dye was found.

QUININE CATION EXCHANGE resin compounds have been used for the indirect measurement of gastric acidity (1–4). While the tubeless technique has marked patient acceptance, the determination in urine of the quinine (5) which gastric acid has eluted is laborious (6–9). At the present time four techniques for the separation of the quinine from the urine are in use, either by means of water-immiscible solvents (7–9), or by ion exchange resin (1). Ion exchange compounds with indicators other than quinine might provide for direct visual determination in urine of the indicator without previous separation.

We undertook a systematic *in vitro* investigation of the elution of indicator materials from ion exchange resins,[1] especially by dilute acids and salt solutions to guide in the choice of possible new resin-dye materials. The indicators were selected for their readily recognizable color. Other considerations in the choice of indicators were toxicity, metabolism, and excretion pattern.

There are 8 possible classical types of resin indicator compounds. These consist of combinations of strong and weak acid resins with strong and weak basic dyes; or strong and weak basic resins with strong and weak acid dyes (Table IV, columns 1–6). Intermediate types of some ion exchange resins are also available and several of these were tested.

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[1] The resins used in this investigation were generously supplied by Chemical Process Company (Duolite®); Dow Chemical Company (Dowex®), and Rohm and Haas (Amberlite®).

EXPERIMENTAL

Batch preparation rather than column preparation of indicator on resins was used since this technique gives homogeneity of product at various concentrations of indicator and more closely parallels gastric elution. A typical procedure for 1 of 21 compounds prepared is presented.

A cation exchange resin in the hydrogen form was washed with distilled water by decantation. A weighed amount of the dye was dissolved in water and added to the resin. The mixture was stirred for one and one-half to two hours with dropwise addition of 0.1 *N* NaOH to maintain the mixture at *p*H 5. At the end of this time the resin compound was filtered, washed with water, and air dried. The amount of dye remaining in the solution and washings was determined, and the amount of dye adsorbed on the resin was calculated by difference. Appropriate modifications of the technique were made when necessary; in the case of basic resin and acid dye compounds the mixture was held at *p*H 8 by the addition of hydrochloric acid. Resin dye compounds were prepared using methylene blue, 2,6-diamino-3-phenylazopyridine and quinine as the cation indicators, and phenolsulfonphthalein and amaranth as the anion indicators. Tables I–IV indicate the resins used with each of these indicators.

In vitro elution studies were made of each of the 21 compounds. The eluting solutions used were 1 *N* HCl, 0.1 *N* HCl, and Ringer's solution. In each case 0.5 Gm. of resin compound in 50 ml. of solution was placed on a mechanical shaker for one-half hour. The amount of dye eluted was determined quantitatively on a Beckman model DU spectrophotometer. The elution data are expressed as mg of dye eluted per Gm. of compound in 100 ml. of solution and are presented in Tables I–IV.

Criteria for evaluation of a compound were both the amount of dye eluted from the resin by the three solutions above, and the ratio of indicator eluted by 0.1 *N* HCl to that eluted by Ringer's solution. The latter is used to estimate the effect of cations other than hydrogen in eluting the dye from the resin. The acidity range between 0.1 *N* HCl and Ringer's solution (*p*H 1–6.7) includes that which is found in human gastric juice. The elution ratio would be favorable if it approached 20:1 for this would permit the assumption that the preponderance of dye released *in vivo* at the intermediate *p*H ranges of the gastric contents is due to hydrogen ion activity in the stomach and not due to the incidental presence of other cations.

In terms of elution ratio, of the 8 possible combinations the compound type that most nearly conforms to the above stated criteria is a combination of a weak acid resin and a strong base indicator. The Amberlite® IRC 50-quinine compound possessed the highest elution ratio (18:1) as well as an adequate amount of indicator eluted. This compound has been clinically evaluated (1–4).

Other compounds which permitted adequate elution and had significant but less desirable elution ratios were Amberlite® XE 96–methylene blue (8:1); Duolite® CS 100–methylene blue (7.5:1), and Amberlite® IRC 50–2,6-diamino-3-phenylazopyridine (7.6:1). The amounts of dye eluted by 0.1 *N* HCl were 3.7, 0.3, and 16.4 mg., respectively. Amberlite® IRC 50–2,6-diamino-3-phenylazopyridine was selected for further study.

Eleven subjects, previously found to be achlor-

TABLE I.—COMPOSITION AND ELUTION CHARACTERISTICS OF METHYLENE BLUE-CATION EXCHANGE RESIN COMPOUNDS

Resin	Type	Dye/Gm. Compd., mg.	mg. Dye Eluted,[a] 1 *N* HCl	0.1 *N* HCl	Ringer's Soln.
Dowex 50X1	Sulfonic acid, strong 1% cross-linked	132.0	1.42	0.29	0.38
Dowex 50X8	Sulfonic acid, strong 8% cross-linked	31.0	0.07	0.01	0.03
Amberlite[b] XE 96	Carboxylic acid, weak	26.4	2.26	3.68	0.47
Duolite C3	Omega sulfonic acid	7.7	0.01	0.004	0.001
Duolite C20	Nuclear sulfonic acid	18.0	0.04	0.002	0.02
Duolite C25	Nuclear sulfonic acid	33.4	0.12	0.01	0.04
Duolite C62	Phosphonous acid	17.0	1.41	0.54	0.22
Duolite C63	Phosphonic acid	7.7	0.34	0.20	0.07
Duolite CS100	Carboxylic acid, weak	16.2	0.23	0.30	0.04

[a] Mg. of dye eluted from 1 Gm. compound in 100 ml. of solution in thirty minutes.
[b] Has been reported by Segal (4).

TABLE II.—COMPOSITION AND ELUTION CHARACTERISTICS OF 2,6-DIAMINO-3-PHENYLAZOPYRIDINE-CATION EXCHANGE RESIN COMPOUNDS

Resin	Type	Dye/Gm. Compd., mg.	mg. Dye Eluted,[a] 1 *N* HCl	0.1 *N* HCl	Ringer's Soln.
Dowex 50X1	Sulfonic acid, strong 1% cross-linked	140.0	15.93	3.04	3.36
Dowex 50X8	8% cross-linked	29.0	1.50	0.16	0.23
Dowex 50X12	12% cross-linked	26.7	1.25	0.20	0.50
Duolite C62	Phosphonous acid	28.3	5.14	1.53	0.18
Amberlite[b] IRC50	Carboxylic acid, weak	32.0	17.53	16.44	2.16

[a] Mg. of dye eluted from 1 Gm. of compound in 100 ml. of solution in thirty minutes.
[b] Has been reported by Segal (4).

TABLE III.—COMPOSITION AND ELUTION CHARACTERISTICS OF PHENOLSULFONPHTHALEIN-ANION EXCHANGE RESIN COMPOUNDS

Resin	Type	Dye/Gm. Compd., mg.	mg. Dye Eluted,[a] 1 *N* HCl	0.1 *N* HCl	Ringer's Soln.
Amberlite[b] IR400 OH	Quaternary ammonium, strong	14.7	2.21	0.34	0.12
Amberlite XE58	Polyamine, weak	8.1	3.70	0.52	0.21
Amberlite XE58 HCl	.....	7.5	5.83	1.78	0.46

[a] Mg. of dye eluted from 1 Gm. of compound in 100 ml. of solution in thirty minutes.
[b] Has been reported by Segal (4).

TABLE IV.—SUMMARY OF COMPOSITION AND ELUTION CHARACTERISTICS OF REPRESENTATIVE RESIN INDICATOR COMPOUNDS

Resin	Type	Strength	Indicator	Type	Strength	Ind./Gm. Compd., mg.	Indicator[a] Eluted By 1 *N* HCl	0.1 *N* HCl	Ringer's Soln.
Dowex 50X1	Sulfonic acid, 1% cross-linked cation exch.	Strongly acidic	2,6-diamino-3-phenylazopyridine	Base	Weak	140.0	15.93	3.04	3.36
Amberlite IRC50	Carboxylic acid	Weakly acidic	2,6-diamino-3-phenylazopyridine	Base	Weak	32.0	17.53	16.44	2.16
Dowex 50X12	Sulfonic acid 12% cross-linked	Strongly acidic	Quinine	Base	Strong	22.2	0.007	0.004	0.003
Amberlite IRC50	Carboxylic acid	Weakly acidic	Quinine	Base	Strong	16.4	0.92	3.55	0.20
Amberlite IR400 OH	Quaternary ammonium anion exch.	Strongly basic	Phenolsulfonphthalein	Acid	Weak	14.7	2.21	0.34	0.12
Amberlite XE58	Polyamine	Weakly basic	Phenolsulfonphthalein	Acid	Weak	8.1	3.70	0.52	0.21
Amberlite IR400 OH	Quaternary ammonium	Strongly basic	Amaranth	Acid	Strong	23.0	0.0	0.003	0.01
Amberlite XE58	Polyamine	Weakly basic	Amaranth	Acid	Strong	17.4	0.05	0.026	0.034

[a] Mg. of indicator eluted from 1 Gm. of compound in 100 ml. of solution in thirty minutes.

hydric by both intubation and CEIQ[2] tubeless tests, were administered 1 Gm. of the compound under the usual conditions of tubeless tests. The range of excretion of 2,6-diamino-3-phenylazopyridine in a two-hour period in these subjects (0.1–5.6 mg.) indicated considerable gastric elution by cations other than hydrogen.

Resin phenolsulfonphthalein compounds (strong and weak base resins combined with a weak acid dye) gave limited range or limited elution ratios (3:1) and were not considered further.

A strong acid resin-weak base dye compound (as typified by Dowex® 50X1—2,6-diamino-3-phenylazopyridine) was found unsuitable, as this compound showed more elution by Ringer's solution than by 0.1 *N* HCl.

In addition, virtually no elution of indicator by any of the three eluting solutions used was obtained from the following types: (*a*) strong acid resin-strong base dye (Dowex® 50X12—quinine); (*b*) strong base resin-strong acid dye (Amberlite® IR 400 OH—amaranth); (*c*) weak base resin-strong acid dye (Amberlite® IR 400 OH—amaranth). The lack of elution of indicator from these compounds shows that they would not be suitable as indicators of gastric acidity, as we would not expect any release of dye to occur in the *p*H range of the gastric contents.

[2] Cation Exchange Indicator Quinine—The Stuart Company, Pasadena, Calif.

SUMMARY[3]

1. A method typical of the preparation of several resin dye compounds is given.

2. An improved *in vitro* evaluation technique is outlined.

3. Representative combinations of resins and indicators were prepared and evaluated.

4. A weak cation exchange resin combined with a strong base indicator was shown to be most nearly suitable.

5. Carboxylic cation exchange resins are suitable for use in tubeless tests for gastric acidity. No suitable dye was found.

[3] Since this work was completed, Segal (10) has prepared and used clinically an Azure A resin compound which falls into the weak acid resin-strong base indicator (dye) category established as suitable by our *in vitro* studies.

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Inhibition of Hydrolysis of Esters in Solution by Formation of Complexes*

II. Stabilization of Procaine with Caffeine

By LEON LACHMAN, LOUIS J. RAVIN,† and TAKERU HIGUCHI

Investigation of the procaine ion–caffeine system has shown that the hydrolytic rate of the ester component is reduced in presence of the xanthine and that this is probably due to formation of a molecular complex between the drug and caffeine. Although this behavior is in accord with previous findings on the benzocaine–caffeine system, evidence seems to indicate that the stoichiometry of the complexing reaction is different. Unlike the one to one complex apparently formed by benzocaine each procaine ion appears to bind two caffeine molecules.

IN THE PRECEDING PAPER of this series (1) it was shown that the rate of hydrolysis of benzocaine in aqueous solution was substantially inhibited by addition of caffeine. The effect was correlated with the formation of complexes between the two compounds. The present investigation is concerned with a similar study on the possible influence of caffeine on the hydrolytic rate of procaine hydrochloride in neutral aqueous solutions. Our experimental findings indicate that the procaine ion also formed a complex with caffeine which cleaves at an insignificant rate as compared to procaine hydrochloride itself.

COMPLEXING BEHAVIOR OF PROCAINE ION–CAFFEINE SYSTEM

Because of the similarity in structure of procaine and benzocaine it is reasonable to assume that a certain degree of complexing may take place between the diethylaminoethyl derivative and caffeine. Apparently no previous work has been done, however, on the complexing tendencies of the ionic species, the form in which the drug is most commonly used in pharmaceutical preparations. Evidence supporting the existence of such a reaction is presented at this time.

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† Fellow, American Foundation for Pharmaceutical Education.

The extent and nature of the complexing reaction was determined following the partition method of Higuchi and Zuck (2). Since procaine ion is essentially insoluble in hydrocarbon solvents, especially in aliphatics, the degree of complexing was followed by determining the caffeine concentration in such organic liquids brought into equilibrium with aqueous solutions of the two reactants.

Unfortunately, as previously suggested (1) and as experimentally determined by Guttman and Higuchi (3), the xanthine exists substantially in aqueous solutions as dimer and probably as tetramer in addition to the usually accepted monomeric species. This behavior is reflected in the partition coefficient plot shown in Fig. 1 of the pure xanthine between water and an immiscible organic layer composed of thirty per cent benzene and seventy per cent isooctane (v/v). In such a system it can be assumed for practical purposes that the concentration of caffeine in the organic layer is a true reflection of only the monomeric caffeine species in the water layer. It is evident, furthermore, that the partition coefficient in the very dilute ranges, which appears to extrapolate to a definite value at zero caffeine concentration, represents the real partition coefficient of the monomeric form alone. From Fig. 1 the partition coefficient of the monomeric form is 20.0 at 30°. Despite the complex nature of the caffeine system in water, it is thus possible, if we assume that the partition coefficient is not seriously affected by low concentrations of other species, to obtain a measure of monomeric caffeine concentration in water by knowing its concentration in the organic layer.

It can be seen from Table I that the caffeine con-

TABLE I.—EQUILIBRIUM CONSTANTS AT VARIOUS CONCENTRATIONS OF CAFFEINE CALCULATED FROM EQUATION 2

Original Concn. of Caffeine in Water $\times 10^3$ Moles/L.	Concn. of Procaine HCl in Water $\times 10^3$ Moles/L.	Concn. of Caffeine in Organic Layer $\times 10^3$ Moles/L.	Concn. of Caffeine in Water Layer $\times 10^3$ Moles/L.	Total Concn. of Free Caffeine in Water and Organic Layer $\times 10^3$ Moles/L.	Concn. of Complex $\times 10^3$ Moles/L.	Concn. of Monomeric Caffeine $\times 10^3$ Moles/L.	Equilibrium Constant A[a] Liter^2/Moles^2
51.50	0	1.50	50.0	51.50	0.0	30.0	
	51.54	1.05	28.4	29.4	22.1	21.0	620
	58.72	1.02	27.0	28.0	23.5	20.4	600
	73.52	0.96	25.2	26.2	25.5	19.4	560
30.90	0	1.07	29.8	30.90	0.0	21.4	
	14.93	0.97	25.6	26.6	5.2	19.4	560
	21.91	0.96	23.0	23.9	7.2	18.0	610
	29.31	0.83	20.9	21.7	9.2	16.6	670
	36.76	0.81	20.3	21.1	9.8	16.2	590
	43.96	0.88	19.2	20.0	10.9	15.4	600
	51.30	0.74	18.1	18.8	12.0	14.8	610
	58.64	0.72	17.6	18.3	12.5	14.4	580
	58.65	0.70	17.2	17.9	12.8	14.0	620
	66.00	0.66	16.0	16.7	14.1	13.2	690
	73.28	0.65	15.6	16.2	14.7	13.0	660
	73.46	0.65	15.6	16.2	14.7	13.0	650
61.90	0	1.84	60.1	61.90	0.0	36.8	
	29.47	1.40	43.4	44.8	17.2	28.0	530
	44.00	1.26	37.0	38.3	23.6	25.2	580
	58.78	1.16	32.5	33.7	28.4	23.2	590
	73.67	1.06	27.0	28.1	32.2	21.2	620
103.00	0	2.26	100.7	103.00	0.0	45.2	
	7.49	2.15	93.5	95.6	7.4	43.0	580
	14.77	2.06	85.5	87.6	15.4	41.2	640
	29.37	1.88	80.9	82.8	29.8	37.6	730
	36.66	1.79	71.5	73.3	35.9	35.8	750
	43.94	1.74	65.4	67.1	39.6	34.8	680
	51.48	1.69	62.6	64.3	42.1	33.8	610
	58.62	1.66	49.2	60.9	46.3	32.4	620
	73.36	1.47	47.0	48.5	54.6	29.4	680

[a] Average A = 620.

centration in the organic layer decreases with an increase in concentration of procaine hydrochloride in the system. This is evidently due to the formation of water-soluble complexes between caffeine and procaine hydrochloride. The equilibrium constants, A, for the interaction between caffeine and procaine hydrochloride, were calculated by assuming that the following reaction took place:

$$2\ \text{Caffeine} + \text{Procaine HCl} \rightleftharpoons \text{Procaine HCl-2 Caffeine} \quad (1)$$

The equilibrium constant for this reaction is then:

$$A = \frac{[\text{procaine HCl-2 caffeine}]}{[\text{caffeine}]^2\,[\text{procaine HCl}]} \quad (2)$$
monomer

The equilibrium constants thus determined are given in Table I showing that they are effectively independent of the procaine hydrochloride concentration at any given concentration of caffeine.

With the partition coefficient of caffeine known the degree of interaction betweeen caffeine and procaine hydrochloride can be calculated on the basis of Eq. 2.

A sample calculation of one of the constants is given below.

Experimentally determined molar concentration of caffeine in organic layer = 10.6×10^{-4}

Partition coefficient value for a 10.6×10^{-4} molar concentration of caffeine in organic layer (from Fig. 1) moles/liter = 27.0

Therefore, the molar concentration of free caffeine in the water layer is: $10.6 \times 10^{-4} \times 27.0 = 2.86 \times 10^{-3}$. Molar concentration of complex is: Initial concentration of free caffeine − experimentally determined free caffeine in water and organic layer, $61.90 \times 10^{-3} - 28.6 \times 10^{-3} - 1.06 \times 10^{-3} = 32.2 \times 10^{-3}$. Original molar procaine hydrochloride concentration = 73.67×10^{-3}. Therefore, the molar concentration of free procaine hydrochloride is $73.67 \times 10^{-3} - {}^1/_2(32.2 \times 10^{-3}) = 57.60 \times 10^{-3}$. Calculated molar concentration of monomeric caffeine assuming a constant partition coefficient of 20 is $20 \times 10.6 \times 10^{-4} = 21.2 \times 10^{-3}$.

Therefore, A, the equilibrium constant for complex formation (Equations 1, 2) is:

$$\frac{(16.1 \times 10^{-3})}{(21.2 \times 10^{-3})^2(57.6 \times 10^{-3})} = 623\ \text{liter}^2/\text{moles}^2$$

In Fig. 2, the amount of procaine hydrochloride-2 caffeine complex formed from varying amounts of procaine hydrochloride for several concentrations of caffeine is given. All the observed results can be predicted with a reasonable amount of accuracy if the A for Eq. 2 is taken to have an average value of

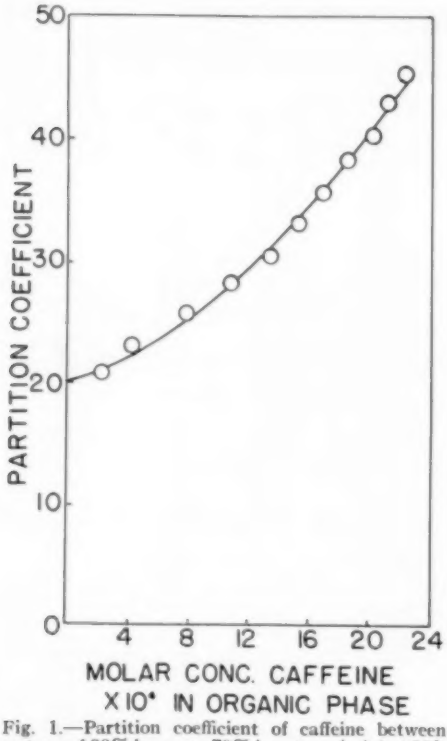

Fig. 1.—Partition coefficient of caffeine between water and 30% benzene–70% isooctane (v/v) at 30°.

620. Deviations from the constant A values are due to the fact that caffeine exists in a polymeric form in the aqueous solution. The most likely factor accounting for these deviations arises from the inability to determine at what concentration of caffeine the polymer exists in equilibrium with monomer.

CHEMICAL KINETIC STUDY

In order to determine the effect of the complexing action of caffeine on the rate of hydrolytic breakdown of procaine hydrochloride, the chemical kinetics of the hydrolytic reaction was studied experimentally in the absence and in the presence of the complexing agent. Higuchi, *et al.* (4), have studied the kinetics of the hydrolysis of procaine hydrochloride. Their results indicate that the rate of hydrolysis over a range of *p*H concentrations was first order with respect to the ester concentration.

The results of a rate study on the hydrolytic breakdown of procaine hydrochloride in water in the absence of and in the presence of caffeine are shown in Fig. 3 and in Table II.

These hydrolytic reactions were carried out in water at 30°. The solutions were adjusted to a *p*H of 7 with phosphate buffer prior to starting the degradation study.

The rate of appearance of *p*-aminobenzoic acid at different concentrations of caffeine in phosphate buffer solution is shown in Fig. 3. The apparent rate constants and half-lives were calculated from these data by the use of a pseudo first order rate equation.

TABLE II.—THE INFLUENCE OF CAFFEINE ON THE HALF LIFE OF PROCAINE HYDROCHLORIDE IN A PHOSPHATE BUFFER SOLUTION AT *p*H 7 AND AT 30°

Caffeine Used, (%)	Half life, Hr.
0.0	935
0.5	1135
1.0	1490
1.5	1689
2.0	1901
2.5	2021

The half life obtained at zero concentration of caffeine is in reasonable agreement with the work of Higuchi, *et al.* (4). The influence of caffeine on the hydrolysis rate of procaine hydrochloride can be readily seen from Table II. It is apparent that the rate of ester breakdown is strongly retarded by the addition of caffeine. This can be ascribed only to the slower rate of breakdown of the complexed ester as compared to the free procaine hydrochloride.

In order to calculate the rate constant for the complexed ester a mathematical expression similar to the one used in our previous publication (1) has been derived. It can be represented as follows:

$$(A[\text{caffeine}]_m^2 + 1)\frac{1}{t_{1/2}} - \frac{1}{t_{(1/2)_f}} = \frac{1}{t_{(1/2)_c}} \times \frac{}{A[\text{caffeine}]_m^2} \qquad (3)$$

where

$t_{1/2}$ = half life for the degradation of procaine hydrochloride in presence of caffeine

$t_{(1/2)_c}$ = half life for the degradation of procaine hydrochloride in the form of the complex

$t_{(1/2)_f}$ = half life for the degradation of free procaine

$[\text{Caffeine}]_m$ = concentration of monomeric free caffeine

A = effective equilibrium constant for the complex formation

Since the value of the effective equilibrium constant A is approximately known from our study of the complexing behavior of caffeine with procaine hydrochloride, it was possible to estimate the k_c or $1/t_{(1/2)_c}$ from any rate value obtained for the degradation of procaine hydrochloride in a solution containing caffeine. For the system containing 2% caffeine it was found that the k_c was approximately zero. From this and other calculations it appeared that the reaction rate of the complexed ester was, at the most, extremely small as compared to that of the free form. Thus for all practical purposes equation 3 reduces to:

$$(A[\text{caffeine}]_m^2 + 1)\frac{1}{t_{1/2}} = \frac{1}{t_{(1/2)_f}} \qquad (4)$$

This low reactivity of the complex is even more evident in Fig. 4 where the per cent free procaine hydrochloride at various concentrations of caffeine have been plotted against the reciprocal half lives of the solutions. The straight line passing through the origin again shows that the reaction rate of the

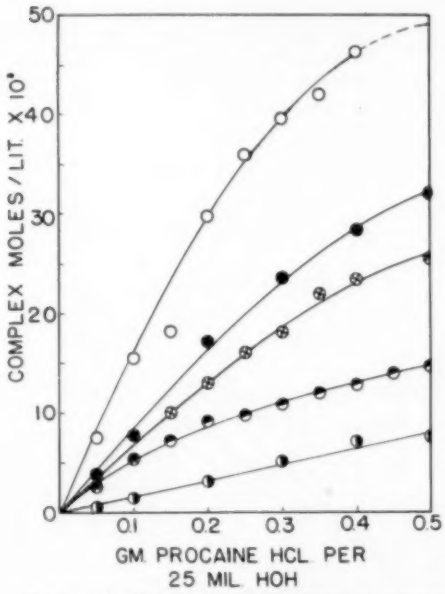

Fig. 2.—A plot of the concentration of complex formed against procaine hydrochloride concentration: ○—0.500 Gm. of caffeine in 25 ml. of aqueous phase; ●—0.300 Gm. of caffeine in 25 ml. of aqueous phase; ⊗—0.250 Gm. of caffeine in 25 ml. of aqueous phase; ◒—0.150 Gm. of caffeine in 25 ml. of aqueous phase; ◑—0.100 Gm. of caffeine in 25 ml. of aqueous phase.

hydrolysis is directly proportional to and a function only of the concentration of free procaine hydrochloride in the solution. The per cent free procaine hydrochloride in the solutions was calculated as follows:

$$\%\ \text{Free Procaine Hydrochloride} = \frac{1}{(A[\text{caffeine}]_m^2 + 1)} \times 100 \quad (5)$$

A correlation of results using various percentages of caffeine is presented in Table III.

It can be seen from Table III that the calculated half lives on the basis, (*a*) that the complexed drug did not degrade and (*b*) that the stability constant of the complex was effectively equal to 620, were in reasonable agreement with the experimentally determined values.

TABLE III.—COMPARISON OF EXPERIMENTAL AND CALCULATED RESULTS AT 30°

Caffeine, %	Free Procaine HCl, %n (Based o $A = 620$)	Exptl. $t^1/_2$ (hr.)	Calcd. on Basis ($A = 620$) $t^1/_2$ (hr.)
0	100.0	935	...
0.5	87.7	1135	1064
1.0	65.7	1490	1421
1.5	58.2	1689	1605
2.0	49.3	1901	1889

The influence of caffeine on the hydrolysis rate of procaine hydrochloride in a phosphate buffer solution of a *p*H of 7 and at 60° is shown in Fig. 5 and in Table IV.

TABLE IV.—THE INFLUENCE OF CAFFEINE ON THE HALF LIFE OF PROCAINE HCL IN PHOSPHATE BUFFER SOLUTION AT A *p*H OF 7 AND AT 60°

Caffeine Used, %	Half Life, Hr.
0.0	26.4
1.0	34.6
2.0	45.8
3.0	52.8
4.0	60.3
5.0	70.8

It can be seen that the half life of procaine hydrochloride is increased approximately threefold by the addition of 5% caffeine. Although the complexing behavior of caffeine with procaine hydrochloride was not studied at 60°, it is believed that the reaction would be similar to that at 30°.

The results of this study again show, as did our previous study on benzocaine (1), that the complex does not appear to undergo any detectable degradation. This further suggests the usefulness of complex formation as an aid in the preservation of many aqueous pharmaceutical preparations. Thus at 2.5% caffeine concentration and at 30° approximately $2^1/_2$-fold increase in the half life of procaine

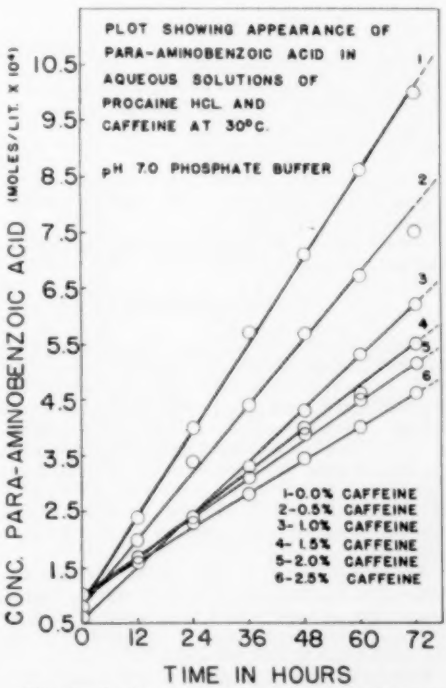

Fig. 3.—A plot showing the effect of caffeine on the rate of hydrolytic degradation of procaine hydrochloride at 30° and at *p*H 7 (phosphate buffer).

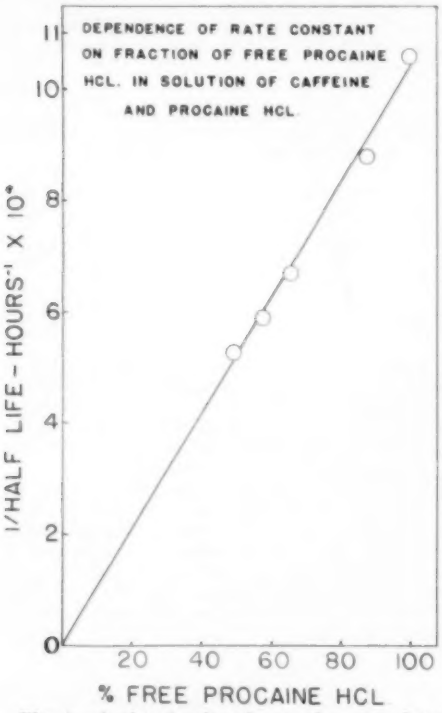

Fig. 4.—A plot showing the reaction rate of the hydrolysis to be directly proportional and a function only of the concentration of free procaine hydrochloride in solution.

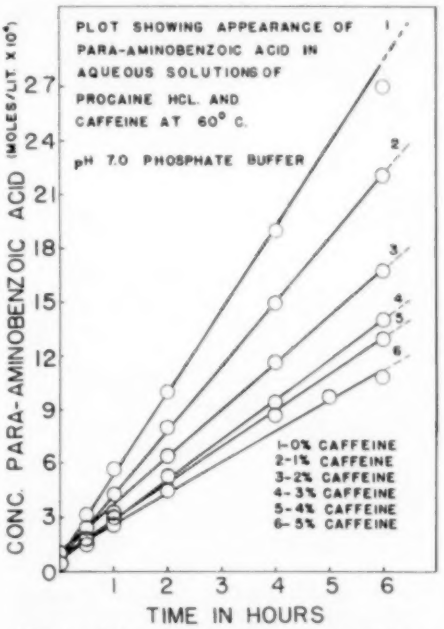

Fig. 5.—A plot showing the effect of caffeine on the rate of hydrolytic degradation of procaine hydrochloride at 60° and at *p*H 7 (phosphate buffer).

hydrochloride was induced by the addition of the complexing agent. Also, at 5% caffeine concentration and at a temperature of 60° approximately threefold increase in the half life of procaine hydrochloride was induced.

EXPERIMENTAL

Reagents.—Procaine HCl, U. S. P., recrystallized from 95% ethyl alcohol m. p. 155–156°; caffeine, U. S. P., recrystallized from water m. p. 235–237°; *p*-aminobenzoic acid, analytical grade (Eastman Kodak) recrystallized from 95% ethyl alcohol, m. p. 187–188°; silicic acid (Mallinckrodt Chromatography Grade) was used to prepare the columns; diisopropyl ether that was purified as described in the previous report (1); benzene, analytical grade; isooctane (Matheson, Coleman & Bell); distilled water.

Apparatus.—Pyrex glass chromatographic columns 45 cm. long and 2 cm. in diameter; a tight fitting glass plunger to pack the columns; Beckman Model DU Spectrophotometer; constant temperature bath set at 30.0±0.05° and at 60°±0.05°.

Chromatographic Columns.—The columns were packed according to the procedure of Higuchi and Patel (5). In preparing the slurry, 0.01 *N* sulfuric acid was used as the internal phase and Skellysolve®-C as the external phase.

Procedure

Complexing Behavior of Procaine Ion–Caffeine System.—The method employed was essentially the same as that of Higuchi and Zuck (2, 6) with the following variations. The organic layer in this system consisted of a solution of 30% benzene and 70% isooctane (v/v). The constant temperature bath was set at 30°. Suitable aliquot portions were pipetted from the organic layer and subsequently assayed for caffeine content by spectrophotometric analysis at 277 mμ. Several determinations were made at constant caffeine concentrations of 0.100 Gm., 0.150 Gm., 0.250 Gm., 0.300 Gm., and 0.500 Gm., while the procaine hydrochloride concentration was varied from 0.050 Gm. to 0.500 Gm.

Chemical Kinetic Study.—The analytical method employed in this investigation was similar to the one used in our previous study (1). The feasibility of the chromatographic technique for this purpose is evident from the chromatogram in Fig. 6. A weakly acidic (0.01 *N*) sulfuric acid solution was employed as the internal phase in this study to suppress any conversion of procaine hydrochloride to procaine base when the sample was added to the column. The procedure of this kinetic study varies appreciably from our previous study, in the following manner.

Accurately weighed quantities of caffeine were placed into 100-ml. volumetric flasks. To the caffeine in the flasks approximately 90 ml. of phosphate buffer was added and the flasks placed into a thermostatically controlled constant temperature bath. When the flask content had reached the

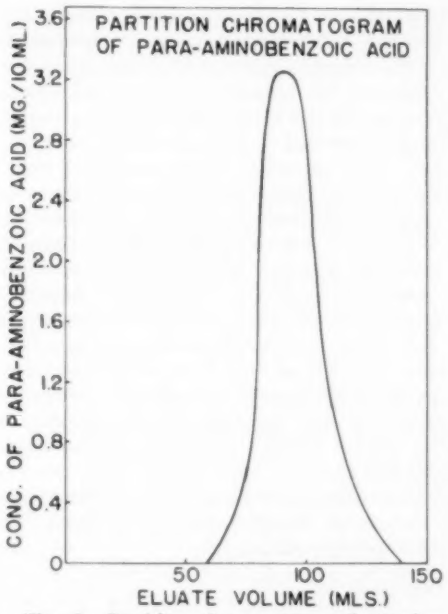

Fig. 6.—Partition chromatogram of *p*-aminobenzoic acid as eluted with diisopropyl ether from an aqueous solution of procaine hydrochloride, *p*-aminobenzoic acid and caffeine. The *p*-aminobenzoic acid was eluted from aqueous solution by the "slurry method" referred to in the text.

temperature of the bath, exactly 10 ml. of a 5% procaine hydrochloride solution was added. The flask was then filled to the mark with buffer solution and shaken. The phosphate buffer was added to produce the desired *p*H for the reaction. The progress of the reaction was followed by sampling at certain intervals.

A 10-ml. sample was taken from each flask for assay. The 10-ml. sample removed from the reaction flask was placed into a 25-ml. volumetric flask to which was added accurately a measured amount of 1.0 *M* H_3PO_4. The quantity of phosphoric acid added adjusted the *p*H of the sample to 3. At this *p*H the hydrolysis reaction ceased. Exactly 5 ml. of this aqueous solution was placed on 5 Gm. of silicic acid in a 50-ml. beaker and mixed thoroughly. A slurry was made by adding 30 ml. of Skellysolve®-C. The slurry was then quantitatively transferred to the column, the beaker rinsed with 10 ml. of Skellysolve®-C, and the rinsings were added to the column. When removing the plunger, care was exercised not to remove any adhering slurry. When approximately all of the supernatant liquid had passed into the column, 1 ml. of diisopropyl ether was added and allowed to drain until almost all the supernatant liquid had again passed into the column. Then 1 ml. of diisopropyl ether was again added and allowed to drain as above. Following this step a 50-ml. portion of diisopropyl ether was added, collected in a graduate, and discarded. The next 150 ml. of diisopropyl ether added was collected in a 200-ml. volumetric flask. The flask was then brought up to volume with diisopropyl ether and analyzed spectrophotometrically at 278 mμ for *p*-aminobenzoic acid.

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Study of Glycolic Acid Metabolism in *Bryophyllum Calycinum* Salisb.*

By HERBERT LIEBERMAN,† EGIL RAMSTAD, and JOHN E. CHRISTIAN

Labeled glycolic acid was found not to be metabolized into isocitric acid under the described experimental conditions (dark metabolism following exposure of the plant to light). The following labeled compounds were detected: peptides, serine, glycine, glyceric acid, alanine, arginine, and histidine. Peptides are early reaction products of glycolic acid.

EVIDENCE is accumulating to show the operation of Krebs tricarboxylic acid cycle in plants (1); not all the acids found in plants have been associated with this respiratory cycle, nor has the biogenesis of all of them been clearly elucidated (2). Included in this latter group of acids are such commonly occurring plant substances as oxalic, lactic, tartaric, and glycolic acids. Experiments by Burris (3) have indicated the presence of glycolic acid in a number of plants. In fact, Burris (3) postulated that the concentration of glycolic acid may be sufficient to support active respiration in a wide variety of plants.

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The major products of anaerobic, dark metabolism of glycolic acid in *Scenedesmus* were found to be glycine and serine (4). After light assimilation of either 1-C^{14} or 2-C^{14} glycolic acid in *Scenedesmus* the label was found equally distributed in the α and β carbon atoms of phosphoglyceric acid whether 1-C^{14} or 2-C^{14} glycolic acid was the substrate. This latter finding would lead one to believe that glycolic acid is closely associated with photosynthesis.

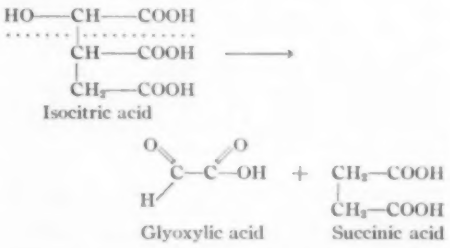
Zelitch and Ochoa (5) isolated from spinach leaves a flavoprotein, glycolic acid oxidase, which catalyzes the oxidation of glycolic acid to glyoxylic acid, and Zelitch (6) revealed in spinach leaves the presence of an enzyme which catalyzes the reduction of glyoxylic to glycolic acid by means of reduced pyridine nucleotide. Zelitch and Ochoa (5, 6) state that the glyoxylic reductase-glycolic oxidase system provides a remarkably effective channel of hydrogen transport from reduced pyridine nucleotides to molecular oxygen and may, therefore, play an important part in the respiration of green leaves.

Tolbert and Cohan (7) presented evidence, using paper partition chromatography, that the major end products from utilization of glycolic acid by barley and wheat plants are glycine, serine, and an unknown.

Since glycolic acid is a common acid in green plants and an effective hydrogen transfer system is involved in its interconversion to glyoxylic acid, further study was initated on the metabolism of glycolic acid in the succulent plants, the acid metabolism of which has been controversial.

The reported high concentration of isocitric acid in the *Crassulaceae* has provoked the minds of scientists for many years. To date Krebs' tricarboxylic acid cycle is the only known pathway for the formation of isocitric acid. According to the equilibrium constant of the reaction citrate → isocitrate, approximately 80 molecules of citric acid should be present for approximately every 16 molecules of isocitric acid (8). Although the reaction equilibrium established between citric and isocitric acids by aconitase is strongly in favor of citric acid accumulation, isocitric acid is reported to represent 50 per cent of the total acidity in *Bryophyllum calycinum* Salisb. (9), and approximately 94 per cent in *Bryophyllum crenatum* Salisb. (10), two species of *Crassulaceae*.

Structurally, isocitric acid can be cleaved to yield succinic and glyoxylic acids, according to the following equation:



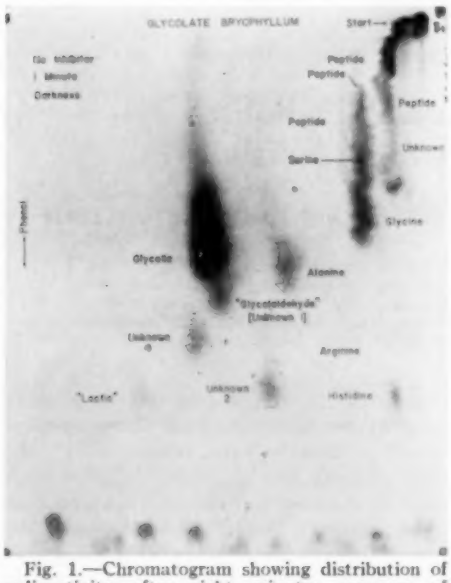


Fig. 1.—Chromatogram showing distribution of radioactivity after eight minutes exposure of *Bryophyllum* leaves to $CH_2OH \cdot C^{14}OOH$.

Fig. 2.—Chromatogram showing distribution of radioactivity after two hours exposure of *Bryophyllum* leaves to $CH_2OH \cdot C^{14}OOH$.

In fact, information appeared in the literature during the course of the present investigation suggesting that bacteria effect a C_2 and C_4 split of citric acid (11–17). Information has recently appeared to suggest a similar cleavage of isocitric acid in bacteria (18). This cleavage does not involve coenzyme A and has as its products succinic and glyoxylic acids.

The reported unusual concentration of isocitric acid in *Bryophyllum* suggests a break in the Krebs acid cycle, and if so, makes this organism excellently suited for the study of an auxiliary respiratory cycle. Certainly the operation of the Krebs tricarboxylic acid cycle in itself does not explain an accumulation of isocitric acid in the succulents. The fact that glycolic acid is present in a number of plants (3) and has been shown to be involved in an effective oxidation-reduction system (5, 6) would lead one to believe that the role of glycolic acid in any respiratory cycle is yet to be revealed, and it was desirable to investigate if glycolic acid in a higher organism such as *Bryophyllum* may play a role in an auxiliary respiratory cycle involving glycolic and isocitric acids.

EXPERIMENTAL

The Sample Size and Physiological Conditions Studied.—All sample leaves were taken from the same clon at approximately 3 p. m. on bright sunny days only. The leaves remained in the sun until they were used for the experimental studies. Disks were bored with a number 3 cork borer from corresponding areas of the blades of several medium-sized leaves. Preliminary experiments indicated that eight disks taken from four different leaves were an adequate and representative sample. Samples were cut from the middle of the blade, adjacent to each side of the midrib. These eight disks were equivalent to approximately 0.3 to 0.4 Gm. of freshly harvested plant.

The 1-C^{14} glycolic acid[1] was imbibed into eight *Bryophyllum calycinum* leaf disks and allowed to be metabolized in complete darkness for eight minutes in one series of experiments, twelve minutes, twenty-two minutes, one hour, two hours and sixteen hours in each of the other experiments. In another experiment of the same series *Bryophyllum* was allowed to metabolize for two days in darkness in the absence of labeled compound. This hunger period was followed by vacuum infiltration of labeled glycolic acid; and then, one hour was allowed for metabolism of the labeled compound in darkness. No respiratory inhibitors were used in the studies with labeled glycolic acid.

Vacuum Infiltration.—The apparatus used for imbibition of the disks with the labeled glycolic acid consisted of a vacuum desiccator attached to a manometer and to the side arm of a suction flask, which acted as a water trap.

Conservation of imbibition fluid was desired in order to maintain the labeled metabolite at a high specific activity. One-half ml. of fluid completely immersed the eight sample disks. The disks were kept submerged by a straight pin attached to a tight-fitting glass tubing that was pressed down by a wire screen. The screen was fitted tightly around the mouth of the test tube and prevented the straight pin holding the disks from being jostled out of the solution during the vacuum infiltration.

Fifty μc. of 1-C^{14} glycolic acid was added to a 2-ml. test tube, which previously had been marked at the $^1/_2$-ml. level with a diamond pencil. One drop of 1 *N* HCl was added, and the acidified solution was brought to the $^1/_2$-ml. level with water.

The plant was maintained at a pressure of 15 to 20 mm. of mercury for seven and one-half minutes. Air was then allowed to seep into the evacuated desiccator until atmospheric pressure was re-established, forcing the solution through the stomata into the intercellular spaces. While the air was seeping into the evacuated desiccator, the whole apparatus with the plant disks was placed in a completely darkened closet for the desired time of metabolism in darkness.

Extraction and Spotting.—After the required time of dark exposure, the eight disks were dropped immediately into 15 ml. of 80 per cent boiling ethanol containing about 1 ml. of 1 *N* HCl in order to help in the extraction of the organic acids. The plant was then boiled in this ethanolic solution for ten minutes in order to destroy all enzymes.

The disks were then placed in a ground-glass tissue homogenizer of 10-ml. capacity containing some HCl-acidified alcohol. The homogenate was filtered through a Gooch filter, and the filtrate transferred quantitatively to a 30-ml. beaker, where the filtrate was evaporated to dryness without use of heat. The dried residue obtained from the homogenate-filtrate was redissolved in 0.3 ml. of water, added in 0.1-ml. fractions. Before the addition of successive portions of water, the sides and bottom of the 30-ml. beaker were scraped with a glass stirring rod to help in redissolving the water-soluble plant constituents.

Each 30-ml. beaker contained the hydro-alcoholic residue derived from eight plant disks, representing approximately 0.4 Gm. of original plant tissue. Two 50-μg. samples of material were pipetted from the suspension in each beaker with the aid of a 1-ml. syringe. The clear supernatant liquid was used whenever possible.

Each 50-μg. sample was added to a spot 13 cm. from both edges of Whatman No. 1 filter paper, 46 cm. by 57 cm. The diameter of the spot was confined to 0.6 cm.

Chromatography and Autoradiography.—These sheets were chromatographed two-dimensionally in chromatocab chambers. The sheets were chromatographed with (*a*) water-saturated phenol in one direction, and with (*b*) a mixture of butanol 4 parts, acetic acid 1 part, and water 1 part in the other direction. The sheets were equilibrated in each chamber for twenty-four hours before introducing each of the solvent systems. The water-

[1] The 1-C^{14} glycolic acid used in these experiments was prepared by the Bio-Organic Group, Radiation Laboratory, University of California, Berkeley, Calif. The acid was received as the calcium salt. It has a specific activity of 5.82 mc. per mg. Calcium 1-C^{14} glycolate was converted to the corresponding acid by use of an ion exchange resin (IR-120(H)).

saturated phenol was run, and then the sheets were air dried for forty-eight hours before introducing the second solvent system. After twenty-four hours of equilibration the second solvent system was introduced. After forty-eight hours of air drying the papers were sufficiently free of solvent to be placed in contact with the No-Screen X-ray Film, 14 inches by 17 inches, emulsion on both sides.

To determine the approximate amount of aqueous extract to be spotted, and the film-contact time with the chromatogram, it was decided to allow the *Bryophyllum* to metabolize vacuum infiltrated, labeled glycolic acid in the dark for two hours, and then to apply varying quantities of the plant extract to the Whatman No. 1 filter papers. The imbibition solution contained 50 mc. per 0.5 ml., and the previously outlined methods for vacuum infiltration, and preparation of plant extracts were followed. It was learned that 50 λ of the 0.3-ml. extract solution gave distinct chromatograms, 46 cm. by 57 cm., after one month of film exposure. The actual contact time used in the experiment with labeled glycolic acid was two months so that added resolution could be obtained.

Identification of the Compounds.—The identities of the metabolites represented in the darkened areas on the autoradiogram were established by comparison of their locations with the locations of known substances similarly chromatographed, and, after elution of the spots, by cochromatographing two-dimensionally the labeled substances with authentic substances. Tentative identification by location was established by comparison of the autoradiogram to a master sheet, prepared from a mixture of authentic substances. Final identification was done by matching the unknown substance to the location of the known suspected substance after two-dimensional cochromatography.

Master Identification Sheet.—A master sheet showing the location of fifty-four commonly occurring plant metabolites after two-dimensional chromatography in the above-described solvents was prepared. Organic acids, amino acids, phospho-compounds, and sugars were chromatographed in two complex mixtures containing approximately thirty different substances.

In two-dimensional chromatography, with chromatographic sheets of 46 cm. by 57 cm., the following concentrations of materials were applied: amino acid, 8–10 μg., except for methionine and glutathione in which case 15 μg. were applied; organic acids, 60 μg., except for α-ketoglutaric and *cis*-aconitic acids of which 100 μg. were applied; sugars, 80–100 μg.; phospho-compounds, 600 μg. of phosphoglyceric acid, and of phosphorylated sugars 1.2 mg. of material was applied.

The color developing reagents used were: Bromcresol green, an acid-base indicator, 0.04 per cent solution in 95 per cent ethanol (19); ninhydrin, an amino acid indicator, 0.2 per cent solution in water-saturated *n*-butanol (19), heated for five to seven minutes at 90°; *p*-anisidine hydrochloride, a coloring agent for aldo- and ketohexoses, and pentoses, 4 Gm. of *p*-anisidine HCl in 10 ml. of water, 10 ml. of ethanol, and the mixture adjusted to a volume of 120 ml. with *n*-butanol (20); molybdic acid, phosphate esters, 5 ml. of 60 per cent (w/w) $HClO_4$, 10 ml. 1 *N* HCl, and 25 ml. of 4 per cent $(NH_4)_2MoO_4$ and brought to a total volume of 100 ml. (21), developed a blue color with phosphate esters after exposure to H_2S for about seven minutes.

Due to slight variation of R_f values as a result of handling and temperature variations, the R_f values in themselves were not used alone as criteria for identification of unknown substances, but, rather, the metabolites were always identified by cochromatography with authentic substances whenever feasible.

The results of the eight-minute metabolism study are illustrated in Fig. 1, and the results of the two-hour metabolism run in Fig. 2. The illustrations are typical of the other autoradiograms obtained from the studies with labeled glycolic acid.

DISCUSSION

An examination of the autoradiograms of the chromatograms obtained from the dark metabolism with labeled glycolic acid revealed that none of the darkened areas matched the R_f values of any of the acids of the Krebs cycle. The compounds that have been identified both by matching R_f values with the master sheet and by two-dimensional cochromatography with known substances are labeled in Fig. 1 and 2. Peptides, serine, glycine, alanine, arginine, histidine, and glyceric acid have been identified. The radioactive spots studied for identification were only those that were heavily labeled. The radioactive areas on the sheet labeled in quotation marks have been identified only by comparison with the master sheet. The amount of activity recovered from the labeled areas of those spots appearing in quotation marks was too low to permit rechromatographing and matching of the material, or, in the case of "glycol aldehyde" or "unknown 1" illustrated in Fig. 1 and 2, no substance was available for comparison.

An identification of "glycol aldehyde" was based on conjecture only. This unknown area is a highly labeled spot which appears to be an early metabolite of glycolic acid (22). An early metabolite of glycolic acid is glyoxylic acid (3, 5, 23). Glyoxylic acid was synthesized by the method described in Beilstein, namely by reduction of oxalic acid with magnesium powder. This reduced solution contained primarily glyoxylic acid (OHC.COOH). The location of glyoxylic acid matched that of glycolic acid and the unknown spot or "glycol aldehyde" spot in the butanol-acetic acid mixture, but glyoxylic acid hardly migrated at all in the phenol-water solvent. Glyoxal (OHC.CHO) was distilled at reduced pressure and chromatographed. Glyoxal was found to match glycolic acid in butanol-acetic acid, but to follow glycolic acid in phenol-water mixture. An analysis of the structures related to glycolic acid that would appear to migrate to the same extent in phenol-water solvent, and to a slightly greater degree in the butanol-acetic acid mixture suggests that unknown 1 may possibly be "glycolic aldehyde" ($HOCH_2.CHO$). This compound is a plausible reduction product of glycolic acid. On rechromatographing unknown 1 or "glycol aldehyde" in the same solvent systems previously used, it was found that roughly $^2/_3$ of the rechromatographed spot did not migrate. One third of the activity matched the R_f value of the original spot. These data are consistent with the fact that after long exposure in

the air, aldehydes polymerize, and polymerization products would generally have low R_f values. Glycolic aldehyde was not available and work is in progress to synthesize this compound so that unknown 1 may be more conclusively identified.

Unknowns 1, 4, and 5 were two-dimensionally cochromatographed with a mixture of succinic, fumaric, malic, and glycolic acids. The location of the radioactivity of each of these unknowns did not correspond to any of the known acid spots.

Unknown 2 was suspected to be methionine, but, by two-dimensional cochromatography with methionine, this possibility was ruled out. Unknown 3 did not have sufficient activity to be used efficiently in cochromatographic testing, and besides, no compounds have been hypothesized which will fit the R_f values of this unknown.

The identity of lactic and pyruvic acids is marked in quotation marks in Fig. 1 and 2. The reason for this is that these substances were not cochromatographed, and, consequently, their identities are presumptive. The radioactivity in these spots was too low, and the number of available sheets with these spots too few, to be eluted and cochromatographed. These spots were identified by the similarity of their R_f values to the known substances of the master sheet.

The point of particular interest for the present problem was the possible formation of isocitric acid from labeled glycolic acid. Isocitric or citric acids do not appear in any of the autoradiograms. To be sure that within some portion of the densely-labeled glycolic acid spot, citric or isocitric acids might not escape observation, a series of experiments was undertaken. It was found by paper partition chromatography that, with a mixture of 10 parts isopropanol, 1 part ammonia, and 1 part water, tricarboxylic acids remain immobile, whereas mono- and dicarboxylic acids move along with the solvent to varying degrees. The glycolic acid spots on the chromatograms were cut in half, an upper and lower portion, in respect to the direction of migration of the butanol solvent system. After elution of these spots, and rechromatographing with the isopropanol-ammonia mixture the activity was located, in each case, in the glycolic acid spot and not in the citric or the isocitric acid spots.

To further substantiate the finding that labeled isocitric acid did not appear in these metabolism studies with labeled glycolic acid, two sheets were sprayed with bromcresol green. Citric and isocitric acids appeared in areas that did not match those of glycolic acid. The nonradioactive spots of citric and isocitric acid were eluted and cochromatographed with authentic substances. On the other hand the heavily radioactive glycolic acid spot gave a hardly perceptible acid reaction. The amount of labeled material was too low to permit its chemical identification. Use of carrier substance along with the unknown radioactive material was, in many cases, the procedure resorted to for identification of the unknowns.

The area of highest activity on all of the autoradiograms from the labeled glycolic acid runs appears near the point of origin. In Fig. 1 and 2 the most radioactive area appears in the three areas marked peptide, near the starting point of the chromatographic paper. These areas of several filter papers were tested with bromcresol green and ninhydrin. An area near the point of origin, which did not correspond to any point of radioactivity on the matched chromatogram, tested, by cochromatography with authentic substance and by matching locations with the master sheet, as oxalic acid. The radioactive streaks gave ninhydrin-positive tests. This finding indicates the absence of labeled oxalic acid and the presence of labeled peptides. This intense radioactive streak of peptide was cut from four such sheets. These separated portions of the sheets were sewed with ordinary needle and white thread to a filter paper lead-strip and chromatographed for four days with the butanol-acetic acid mixture. The four sheets were placed in contact with film for six weeks. The strongly labeled streak separated into three highly labeled bands, indicating the presence of at least three labeled compounds. A portion of one of these filter paper strips was tested with ninhydrin, and the positive ninhydrin areas corresponded to the radioactive bands. It appears as though glycolic acid is not metabolized into oxalic acid, and is quickly converted into early metabolites, which appear to be peptidic in nature.

An examination of the numerous metabolic products of labeled glycolic acid after eight minutes (Fig. 1) and two hours (Fig. 2) of dark metabolism reveals the ease and rapidity of utilization of glycolic acid. The conversion of glycolic acid to glycine, serine, alanine and what was referred to as "glycol aldehyde" is apparent. The almost immediate and most pronounced conversion of the labeled glycolic acid to highly active peptides is particularly striking.

From an analysis of these data it appears as though glycolic acid is not directly hooked up into any of the tricarboxylic acids in *Bryophyllum* in dark metabolism. In this plant some of the major primary products formed from glycolic acid are several different peptides, glycine, and serine. Alanine, arginine, and histidine are later products of glyoxalic acid, a proved metabolic (3, 23), and oxalic acid, hypothesized by many biochemists as a possible metabolic product from glycolic acid metabolism.

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Physical Properties and Pharmacological Activity: Antihistaminics

By NICHOLAS G. LORDI and JOHN E. CHRISTIAN

The ionization constants, solubilities, and relative surface activities of sixteen commercially available antihistaminics are reported. The antihistaminic effect is reviewed and discussed in the light of these properties. The usefulness of solubility as an indicator of toxicity, duration of effect, and local anesthetic potency is emphasized.

THE IMPORTANCE OF physical properties in the interpretation of drug action must not be underestimated. The ability of a drug to combine with a receptor, to accumulate at a particular site of action, to penetrate a membrane, all are dependent in part on such properties as ionization, solubility, and surface activity. No treatment of the mechanism of drug action can be considered complete unless these factors are taken into account.

Structure has not always proven a successful indicator of biological activity. Thousands of compounds of a particular structural type might be synthesized, yet only a few would show desirable clinical features. Although structure determines physical properties in large degree, the knowledge of physiochemical constants provides an avenue of approach to the problem of structure-activity correlation on a finer level. Such an approach could well lead to the more successful prediction of biological activity, both qualitative and quantitative, as well as give an estimate of the toxicity of new compounds prior to pharmacological screening.

The ionization constants, solubilities at *p*H 7.4 and 37.5°, and relative surface activities at *p*H 7.4 of sixteen commercially available antihistaminics were determined. In these respects, little has been done as far as antihistaminics are concerned. Tolstoouhov (1) has correlated the basicity of N′-aliphatic substituted N,N-dimethylethylenediamines with antihistaminic activity. Baltzly, *et al.* (2), have investigated the effect of aromatic substitutions on the ionization constant of cyclizine. Zetler (3) has made a limited study of the surface activity of antihistaminics.

EXPERIMENTAL

Materials.—Antihistaminics[1] were used in the form of the hydrochlorides with the following exceptions: Thephorin® tartrate, Tagathen® citrate, Doxylamine® succinate (and maleate), Pyronil® phosphate, and Trimeton®, Chlor-Trimeton®, and Neo-Antergen® maleates. All salts were recrystallized from absolute alcohol-ether mixtures. Papaverine HCl, U. S. P. XIV, and histamine dihydrochloride, Eastman Kodak, were utilized without prior treatment.

Measurement of Ionization Constants.—A semimicropotentiometric titration method was employed. Ten-ml. aliquots of from 0.002 to 0.01 *M* solutions of antihistaminics were titrated with standard 0.2 *N* KOH until cloudiness appeared in solution. A micrometric gage and a calibrated syringe capable of delivering to ±0.001 ml. was used as a microburet. A Coleman *p*H Electrometer, Model 3D, was utilized for the *p*H measurements. The electrode system, consisting of a Beckman 3-inch calomel electrode and a Coleman No. 3-031 glass electrode, was checked against a saturated solution of potassium acid tartrate (4), *p*H 3.57 ± 0.02 at 25°, and a buffer of *p*H 7.00 before each run. The precision of the *p*H measurements was better than ±0.02.

Calculation of the appropriate constants was made from *p*H values between 10 and 80 per cent of neutralization using, in part, the equations derived by Tolstoouhov (5). In order to avoid confusion, the terminology according to Hammett (6) was adopted.

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[1] The following are gratefully acknowledged for the generous supplies of antihistaminics used in this study: Abbott, Burroughs-Wellcome, Ciba, Hoffmann-La Roche, Lederle, Lilly, Merck, Merrell, Nepera, Parke-Davis, Schering, Sharp and Dohme, Winthrop Laboratories, and Wyeth.

TABLE I.—PHYSICAL PROPERTIES OF SOME COMMERCIALLY AVAILABLE ANTIHISTAMINICS AND OTHER COMPOUNDS

Compound[a]	Antihistaminic Activity Index[b]	pK_{a1}'	pK_{11}'[c]	pK_{a2}'[d]	pK_{12}'	Ionization, %	Solubility Moles/L. pH 7.4, 37.5°	Relative Surface Activity[e]
Ethylenediamine Group								
Pyribenzamine	VI	8.96[g]	5.04	3.92	10.08	98	0.015	1.3
Neo-Antergan	VI	8.92[g]	5.08	4.02	9.98	97	0.012	1.6
Thenylpyramine	II–VI	8.91[g]	5.09	3.66	10.34	97	0.017	0.8
Thenfadil	VI	8.93	5.07	3.94	10.06	97	0.017	0.7
Tagathen	VI	8.42	5.58			91	0.0068	6.3
Neohetramine	II	8.84[g]	5.16	2.08	11.92	96	0.019	1.0
Ethanolamine Group								
Benadryl	III	9.00[g]	5.00			98	0.012	3.2
Doxylamine	III	9.20	4.80			99	0.030	0.6
Ambodryl	V (12)	8.64	5.36			94	0.0022	16.7
Propylamine Group								
Trimeton	VI[f]	9.29[g]	4.71			99	0.031	0.1[f]
Chlor-Trimeton	VI[f]	9.16[g]	4.84			99	0.020	1.3
Other Antihistaminics								
Antistine	II	10.06[g]	3.94			100	0.019	0.5[f]
Chlorocyclizine	VI	7.81	6.19	2.43	11.57	72	0.0010	38.5
Phenergan	IV	8.65	5.35			94	0.0012	17.9
Pyronil	VI (13)	8.77	5.23			96	0.00087	47.6
Thephorin	IV	8.29	5.71			88	0.0019	17.0
Other Compounds								
Histamine	..	9.68	4.32	5.88	8.12	100	...	0.0
Procaine	I	8.98	5.02[h]			98	0.042	0.03[f]
Papaverine	I	6.40	7.60	(14)		9	0.00011	27.0[i]

[a] Classified according to fundamental structural residue.
[b] Notation adopted from that of Leonard and Huttrer (15): I—extremely weak activity; I, II—weakly active; III, IV—active: V, VI—very active; and VI—extremely active. All figures represent *in vitro* activity and are taken from Leonard and Huttrer unless otherwise noted.
[c] $pK_1 = pK_w - pK_a$ where pK_w is 14.00 at 25°.
[d] Second ionization constants of compounds containing two basic nitrogens.
[e] 1/Concn. which gives a surface pressure of 10 dynes times 10^{-3}.
[f] *In vitro* activity.
[g] Tolstoouhov (1) has also reported pK values for these compounds. With the exception of Trimeton and Chlor-Trimeton, none of the values recorded here check with those he has reported. Discrepancies are as much as 1–2 pK units in a few cases (e.g., thenylpyramine—7.64, Benadryl—7.56). Considerations of the effect of structural changes on pK tend to support the values given here.
[h] Krahl, *et al.* (9), report a pK_1' of 5.06.
[i] Calculated from concentrations extrapolated to a surface pressure of 10.

For the equilibrium:

$$BH^+ + H_2O \rightleftharpoons OH_3^+ + B$$

Apparent acidity constant $K_a' = (OH_3^+)[B]/[BH^+]$

Apparent ionization constant $K_i' = [BH^+](OH^-)/[B]$

No attempt at temperature control was made. The temperature of all samples was measured, then the calculated constants corrected to 25°, utilizing the correction factors given by Albert (7). For bases of pK_a' between 8 and 14, 0.02 units were added per each degree above 25; 0.01 units were added for bases of pK_a' between 1 and 7.

Ambodryl®, Phenergan®, chlorcyclizine, Tagathen®, Thephorin®, and Pyronil® were found to be too insoluble. Their pK_a' values were evaluated in 20, 40, 60, and 80 per cent alcohol where possible. The value in water was obtained by extrapolation in a manner similar to that indicated by Hall and Spinkle (8). Neo-Antergan®, Trimeton®, Chlor-Trimeton®, and Doxylamine® were titrated as the maleates, the second inflection point being used for the calculations. Tagathen®, Thephorin®, and Pyronil® bases were titrated with 0.2 *N* HCl. All other compounds were titrated as the hydrochlorides.

The second pK_a' of compounds containing two basic nitrogens was obtained by titration of the salt with 0.2 *N* HCl.

Constants are recorded in Table I with an estimated uncertainty of ±0.05. The pK_a' values obtained by extrapolation from alcoholic solution are uncertain to within ±0.10 (Pyronil®, Thephorin®, and Tagathen® to within ±0.15). All results are an average of two separate determinations.

The per cent ionization to be expected under physiological conditions (37.5°, *p*H 7.4, and ionic strength of 0.15) was estimated. Since an increase in ionic strength serves to increase pK_a and an increase in temperature to decrease pK_a, the two factors were assumed to cancel and the pK_a' which would prevail under physiological conditions set equal to that obtained at 25°.[1]

Per cent ionization (7) = 100/1 + antilog (*p*H − pK_a')

Solubility Determinations.—The free bases were freshly prepared by making a solution of the salt alkaline with ammonia and extracting with ether, washing, and removing the ether under reduced pressure. Their solubility was evaluated in *M*/15 phosphate buffer, *p*H 7.4, which had a calculated ionic strength of 0.17. Preliminary experiments had indicated that the compounds had a negative

[1] A 12° increase in temperature is on the average equivalent to 0.24 *p*H units. Krahl, *et al.* (9), reported the effect of an increase in ionic strength on the pK values of several local anesthetics. For a u. of 0.16, the change in pK_a was on the average 0.20.

temperature coefficient for solubility in the buffer. This was observed to be true for all compounds except Antistine®, procaine, and papaverine.

An excess of base was shaken for one-half hour with buffer in a mechanical shaker at room temperature before equilibrating in a thermostat held at 37.5 ± 0.2°. After fifteen minutes, buffer was added until the resultant cloudy solutions just cleared. They were then equilibrated for an additional hour with occasional shaking. Solid bases[3] (Antistine®, Thephorin®, Phenergan®, procaine, and papaverine) were melted, shaken with hot buffer, and then equilibrated in the thermostat. The solutions were filtered through wetted, coarse sintered glass funnels. Appropriate aliquots were mixed with 5 ml. of 1 N H_2SO_4 and diluted to 50 ml. with distilled water.

All analyses were conducted with the Beckman Model DU spectrophotometer in the region 230–300 mμ. Saturation concentrations were determined in moles/L. from standard concentration-optical density calibration curves. All samples were run in triplicate, the precision being better than ±3 per cent in most cases. Only Pyronil® and Thephorin® were observed to undergo detrimental changes during the period of measurement. Solubilities are recorded in Table I to the nearest 2nd significant figure with an estimated uncertainty of ±10 per cent; in the cases of Pyronil® and Thephorin®, ±20 per cent.

Surface Activity Measurements.—The Du Nouy Interfacial Tensiometer was employed at a room temperature of 23 ± 2°. The surface tension of solutions of all compounds in $M/15$ phosphate buffer adjusted to a pH of 7.4 was measured in the concentration range 0–0.05 M. Depressions in surface tension (surface pressures) were calculated, thus eliminating the need for applying correction factors for the shape of the liquid held up by the ring. The straight lines obtained by log-log plots of surface pressure versus concentration were generally parallel. Results are recorded in Table I in terms of the reciprocal of the concentration required to produce a surface tension lowering of 10 dynes/cm. This value was used as a rough measure of the relative surface activity.

DISCUSSION

Before the properties listed in Table I can be intelligently related to physiological activity, certain limitations inherent in studies of this type must be emphasized. First, only a limited number of compounds were examined, and those, for the most part, successful antihistaminics. Procaine and papaverine were chosen for comparison because they represented two compounds with divergent structures and weak antihistaminic activity in addition to possessing other pharmacological effects in common with antihistaminics.

The second limitation arises from the fact that comparisons with the pharmacological data are difficult, due primarily to a lack of consistency of procedure on the part of the various workers in the field and secondarily to biological variation.

Consideration must also be given to the limitation referred to by Tammelen and Löfgren (16). In essence, correlations between physical properties and physiological activity in homologous series are expected, but give no indication of mechanism of action. Only where correlation is observed between compounds of divergent structure can something be said of mechanism of action.

Interrelations Between Structure and Physical Properties.—Introduction of *para* substituents results in decreases in both pK_a' and solubility (e. g., the following pairs: Pyribenzamine®-Neo-Antergan®, Benadryl® - Ambodryl®, thenylpyramine - Tagathen®, and Trimeton®-Chlor-Trimeton®). Substitution of a thenyl group for a benzyl (2nd order isosters) has little effect on pK_a (e. g., Pyribenzamine®-thenylpyramine). Antistine®, a secondary amine, has a greater pK_a than histamine, a primary amine.

Surface activity tends to parallel the reciprocal of the solubility: i. e., the more insoluble the compounds, the greater the surface activity. None of the properties evaluated are mutually exclusive. Both solubility and surface activity at any given pH are a function of ionization.

The Antihistaminic Effect.—A careful examination of Table I indicates that no apparent correlation exists between any of the physical properties reported and antihistaminic activity. This lack of correlation may be taken as an indication of the high degree of specificity of the antihistaminic effect.

Antihistaminics are generally regarded to act by competing with and displacing histamine from a receptor. This theory was originally proposed by Halpern (17) in 1942 and since then application of the theory of competitive inhibition to the interpretation of pharmacological data (18, 19) has apparently strengthened Halpern's concept. The receptor concept implies specificity of configuration with regard to agonist and antagonist. That the steric factor is important in interpreting antihistaminic action can be seen by examining any table of structure-activity correlations (15). The most powerful evidence for demonstrating specificity of configuration would reside in the observation of differences in degree of activity for optically isomeric compounds.[4] Green (20) provides such evidence. He has shown that the *d*-isomer of N,N dimethyl - 3,3 - dithienyl - 1 methyl - propenamine has greater than two times the activity of the *l*-isomer.

Fleckenstein and Hardt (21) have suggested that antihistaminics achieved their action by reversibly condensing the plasma membrane of the cell, resulting in a decreased permeability and increased stabilization. Data accumulated by Parrot (22) and co-workers on the effect of antihistaminics on the permeability of isolated guinea pig organs to potassium ion tend to support this view. Such an effect, which is nonspecific in character, would show up more strongly on easily excitable tissue such as

[3] It is more significant to compare thermodynamic activities rather than solubilities. If the standard state chosen is the pure liquid (10, 11), solids must be melted in order to have comparable standard states. Correction should then be made for the fugacity change on melting a solid. Such correction necessitates a knowledge of the heat of fusion. However, the correction can be neglected in the case of all solid bases investigated with the exception of papaverine, since they are ionized to an extent greater than 90 per cent. The correction applies only to that portion of material which does not dissolve because of ionization.

[4] It is also interesting to note that the following commercially available compounds are racemic mixtures: Ambodryl,® Doxylamine®, Trimeton®, Chlor-Trimeton®, Phenergan®, chlorcyclizine, and Thephorin®.

nerve (antihistaminics are strong local anesthetics) than on the much less excitable smooth muscle.

That the antihistaminic action must involve a nonspecific component is further indicated by the diversity of compounds which show such action however slight. Examples range from the phenols (23), nitrogen mustards (24), flavones (25), Tweens® (3, 26), lobeline (27), and thioalcohols (28) to calcium (29) and lysolecithin (30). Many of these substances have in common the property of surface activity. At low concentrations they would tend to accumulate on the cell surface and exert a stabilizing effect. They would also be expected to be approximately equally effective in blocking the spasmogenic action of barium and acetylcholine. This is generally true. The weak antihistaminic activity of such compounds as atropine and papaverine as well as the atropine- and papaverine-like actions of antihistaminics might be attributed to the operation of this nonspecific effect.

The question can be asked: In what manner can physical properties be of value in interpreting specific drug action? In this respect the significance of physical properties can be visualized in three ways: in terms of access to the site of action, forces which tend to bind the molecule to the receptor, and persistence of effect.

A qualitative interpretation of access might be made in terms of solubility. Two physical processes which partially define access are diffusion and permeability. The penetrating power of a molecule through a predominantly lipophilic membrane would be related to its degree of nonpolarity and thus to its relative insolubility. On the other hand, the smaller and more soluble a molecule is, the more readily diffusible. The action of the highly soluble histamine on gut is practically instantaneous while the blocking effect of the less soluble antihistaminics involves a time factor. A slow onset of action is especially characteristic of the least soluble compounds, e. g., Pyronil®, chlorcyclizine, etc. The wide discrepancies in apparent activities of antihistaminics on gut as recorded in the literature might be attributed to the occurrence of selective diffusion processes.

Electrostatic and Van der Waals forces are involved in the binding and attraction of the drug to its receptor. That an ionic bond must be involved in the attachment of the drug to the receptor can be readily inferred from existing data, since histamine and the antihistaminics exist predominantly in the form of the cation under physiological conditions. One must conclude from a knowledge of the relative surface activity of these compounds that the electrostatic component outweighs other factors in the attraction of histamine to the receptor, while it is of lesser importance in the case of the relatively highly surface-active antihistaminics. The fact that the antihistaminic activity of 2-haloethylamines is due to the formation of an ethylidinium ion (31), e. g., R $\overline{R'NCH_2CH_2}^+$, is also indicative of the existence of an anionic residue in the receptor.

If the ionic bond were the sole factor which determined the efficacy of the drug receptor combination, then one would expect the degree of activity to be related directly to the pK_a, the stronger bases forming stronger bonds and therefore, showing the greatest activity. However, the lack of correlation between pK_a and antihistaminic activity has also been shown to be the case for histamine-like activity (32). Structural changes in antihistaminics which would tend to make them stronger bases (e. g., quaternization, and changing from tertiary to primary or secondary amines) result in large reductions in activity as well as reducing the effectiveness in which these compounds can engage in attractions of the Van der Waals type and increasing their solubility Changes in structure which tend to increase the effectiveness of these attractive forces are limited by the operation of steric factors. With the exception of introduction of substituents of small size in the *para*-positions of aralkyl rings, such changes usually result in reduced activity.

Solubility may be used as a rough indicator of the magnitude of these short-range forces: the less soluble, the greater the attractive force. The same considerations apply also to an analysis of persistence of effect. Those compounds which show the most prolonged action are generally the most insoluble.

The Local Anesthetic Effect.—Solubilities can be used to estimate local anesthetic potencies (LAP). The theoretical justification for this resides in Ferguson's principle (33). He found that when certain toxic effects were measured at equilibrium, the thermodynamic activity at which substances give equal degrees of physiological activity would be a constant. When dilute solutions are involved, and the standard state selected as the pure substance, under conditions of equipotency, the activity can be taken as equal to the toxic concentration divided by the saturation concentration.

Conduction block in nerves is produced at five per cent of saturation (34). If one assumes that both procaine and the antihistaminics have the same activity for conduction block, the theoretical LAP of antihistaminics relative to that of procaine

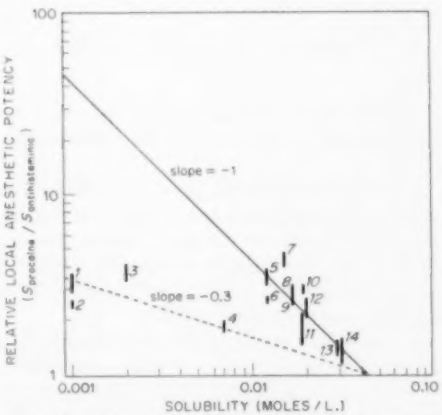

Fig. 1.—Comparison of measured and theoretical local anesthetic potencies of antihistaminics relative to procaine calculated from solubilities in *M*/15 phosphate buffer, *p*H 7.4, at 37.5°: *1*, Phenergan; *2*, chlorcyclizine; *3*, Thephorin; *4*, Tagathen; *5*, Benadryl; *6*, Neo-Antergan; *7*, Pyribenzamine; *8*, Thenfadil; *9*, thenylpyramine; *10*, Antistine; *11*, Neohetramine; *12*, Chlor-Trimeton; *13*, Doxylamine; *14*, Trimeton.

equals the ratio of the solubility of procaine to that of the antihistaminics. However, since under physiological conditions most of the antihistaminics are better than 90 per cent ionized, only a small fraction of the molecules will be available in the physiologically active form which for local anesthesia is the free base (9).

In order to see if such a relationship could be demonstrated, the local anesthetic activities of antihistaminics as reported by Stephenson and Allmark (35) were examined. A log-log plot was made of the ratio of the solubilities of procaine and antihistaminics (referred to *p*H 7.4, ionic strength of 0.17, and 37.5°) and the potencies, referred to procaine as a standard, of 14 antihistaminics recorded on Fig. 1 as lines drawn between the reported confidence limits. Although the potencies were obtained by the intracutaneous wheal method on guinea pigs, compounds of a solubility greater than 0.01 *M* show excellent agreement. The more insoluble compounds show large discrepancies. This is to be expected, since potencies forty times that of procaine are extremely unlikely.

Other Pharmacological Properties.—Since irritancy has been successfully correlated with surface activity of local anesthetics (36), one would expect most of the antihistaminics to be much more irritant than procaine. Limited studies of the irritancy of antihistaminics (37, 38) would seem to substantiate this.

The solubility of a substance is essentially a measure of its availability for exerting physiological activity. It might, therefore, prove useful as a measure of the acute toxicity of antihistaminics. In this respect, it is important to note that of the antihistaminics proved clinically useful, the least toxic are those which are the most insoluble (e. g., Pyronil®, chlorcyclizine, Phenergan®, Ambodryl®, etc.) or the most soluble (e. g., Neohetramine®, Trimeton®, Doxylamine®, etc.).

SUMMARY AND CONCLUSIONS

1. No direct correlation could be made between physical properties and the antihistaminic effect.

2. The antihistaminic effect was discussed in terms of a nonspecific and specific component. The latter, being the dominant factor, was discussed from the viewpoint of access to the site of action, forces which tend to bind the drug to the receptor, and persistence of effect.

3. In the interpretation of the physiological effects of antihistaminics, solubility was shown to be the most important single property. The least soluble antihistaminics are among the least toxic, have the slowest onset of action, and have the most prolonged duration of effect.

4. A method of roughly estimating local anesthetic potencies relative to procaine as a standard was indicated. For compounds of solubility less than 0.01 *M* at *p*H 7.4 and 37.5°, the potency could be set equal to the ratio of the solubility of procaine to that of the antihistaminic; for compounds of solubility between 0.01 and 0.001 *M*, the potency could be set equal to the same ratio in which the solubility of the antihistaminic is raised to the 0.3 power.

5. Correlation of the physical properties of antihistaminics with their other pharmacological properties was limited by the lack of suitable data. Even though the action of a drug may be highly specific, the knowledge of the physical properties would still be of use to the pharmacologist as an aid in the more effective design of his experiments as well as in the interpretation of his results. However, the physical data, no matter how precise, are only as good as the available pharmacological information.

6. Assuming free access to the site of action, correlation should be observed between any given physical property and antihistaminic activity when all other factors are held constant. The antihistaminic effect is dependent on the balance between the nonpolar, polar, and ionic portions of the molecule as modified by its steric configurations.

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Studies on Rauserfia; A Rauwolfia Derivative*

By JOHN W. WARD and LLOYD W. HAZLETON

The increasing use of *Rauwolfia serpentina* and its derivatives in the treatment of hypertension and psychiatric disorders has prompted the reporting of a series of animal studies on Rauserfia, an alkaloidal derivative of rauwoifia, from which the strongly basic alkaloids and ajmaline have been removed. The material causes an immediate and pronounced hypotensive effect following intravenous administration. This action differs from that of reserpine. Acute, subacute, and chronic toxicological investigations indicate a low order of toxicity. Rats tolerated for extended periods of time dosages of Rauserfia which were 140 times the recommended human daily dose.

INTEREST IN the therapeutic usefulness of several species of *Rauwolfia* and its derivatives has mounted rapidly within the last few years. The report of Vakil (1), and later Wilkins and Judson (2), have stimulated an extensive clinical trial of these drugs as antihypertensive agents. Furthermore, recent clinical evaluation of rauwolfia as an adjunct to psychotherapy (Kline (3); Yonkman, *et al.* (4)) has been undertaken. The drug appears to be especially promising in patients who experience anxiety, tension, and hyperactivity. Rauserfia[1] designates a mixture of alkaloids from *Rauwolfia serpentina* from which the strongly basic alkaloids and ajmaline have been removed. It contains 95 per cent total alkaloids, of which 25 to 35 per cent is reserpine. The following investigations were undertaken to evaluate acute and prolonged effects of Rauserfia in animals. The material was supplied under code numbers of RL-1103, 1861-LLA, 2290-LLA, and RLP-237-268.[2]

EXPERIMENTAL

Acute Toxicity.—RL-1103 and 1861-LLA were suspended in 0.5 per cent weight/volume aqueous methylcellulose for purposes of injection. Statistical evaluations of the LD_{50}'s were by the method of Litchfield and Wilcoxon (5), unless otherwise noted, and appear in Tables I and II. *Rats.*—Unstarved male albino rats, weighing about 120 Gm., were injected orally with the drugs. Signs of toxicity and gross pharmacologic effects were in general the same for both materials. Sedation and ptosis occurred within several hours at each dosage and lasted for periods up to six days. Salivation, diarrhea, tremors, ataxia, and hypothermia to touch were also apparent at most doses. Survivors were observed for seven days, sacrificed, and autopsies performed. There were no significant gross pathological findings in any animal; at forty-eight hours there were no significant differences in the calculated LD_{50}'s of 270 and 183 mg./Kg. of RL-1103 and 1861-LLA, respectively. Deaths were observed as late as the fifth day. Latent mortality was accompanied by marked losses in body weight (30–40 Gm.) which suggests that death may have resulted from undernourishment and malnutrition.

Mice.—Unstarved male albino mice, weighing about 20 Gm., were injected intraperitoneally with the drugs. Sedation, ptosis, and diarrhea were apparent within the first few hours following the injection of each compound at doses of 10 to 100 mg./Kg. At higher doses of 464 and 1,000 mg./Kg., death was very rapid, usually occurring within fifteen minutes after injection. Salivation, exophthalmia, irregular respiration, and clonic convulsions usually preceded rapid deaths. Survivors appeared sedated and exhibited ptosis and hypothermia to touch for periods as long as seven days. Many latent deaths occurred from the third through the eighth days. A marked reduction in body weight was evident with latent mortality, suggesting that starvation was responsible. LD_{50}'s based on mortality at seven days were of no practical significance since the very flat slopes indicate a nonspecific type of death. At forty-eight hours the

* Received September 10, 1955, from Hazleton Laboratories, Falls Church, Va.
[1] Manufactured by S. B. Penick and Company, New York, N. Y.
[2] Derived from *Rauwolfia heterophylla*.

LD_{50}'s for both RL-1103 and 1861-LLA were between 100 and 464 mg./Kg. It seems unlikely that deaths within this period would have been influenced by such extraneous factors as undernourishment and malnutrition. Survivors were observed for ten days, sacrificed, and autopsies performed. There were no abnormal gross findings.

Pharmacodynamics.—Six mongrel dogs were used in these studies. The animals were anesthetized by intravenous pentobarbital sodium (30 mg./Kg.) and prepared in the usual manner for recording blood pressure and respiration. Rauserfia was dissolved in several drops of glacial acetic acid and diluted to the required volume with water.

Intravenous administration of the compound in doses of 95 to 250 μg./Kg. caused an immediate and significant (20–73%) lowering of the blood pressure (Fig. 1). Higher doses produced an apparent respiratory depression. It is evident that the acute depressor response to Rauserfia is due to other alkaloids than reserpine, because other studies in this laboratory have indicated that reserpine exerts no such effect, even in doses of 1,000 μg./Kg. Furthermore, it has been demonstrated by Plummer, *et al.* (6), that the hypotensive response to reserpine is delayed, occurring two to three hours after administration.

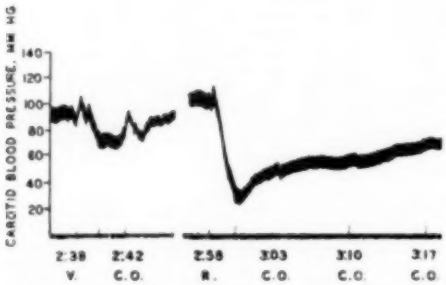

Fig. 1.—Hypotensive effect of Rauserfia, No. 2290-LLA in the dog. *V* = vehicle; *R* = 250 μg./Kg. of Rauserfia; *C.O.* = bilateral carotid occlusion for forty-five seconds.

The gradual abolition of the carotid sinus reflex (C. S. R.) following repeated small doses (10 μg./Kg.) of Rauserfia is presented in Fig. 2. The blood pressure remained relatively stable for a control period of one hour previous to the first injection of Rauserfia. Suppression of the C. S. R. was accompanied by a gradual hypotensive effect, the nadir occurring simultaneously with abolition of the reflex. It appears improbable that disappearance of the C. S. R. resulted entirely from the fall in blood pressure, since subsequent tests demonstrated antagonism when the blood pressure was at normal levels (110 mm. Hg). A total of 190 μg./Kg. of Rauserfia (48 to 67 μg./Kg. reserpine) given over a period of fifty-six minutes was necessary to abolish the C. S. R. Trapold, *et al.* (7), produced a blockade of the C. S. R. in dogs with 500 μg./Kg. reserpine while Bien (8) observed the same effect in cats after 30 μg./Kg. reserpine. The rather slow onset of action which is characteristic of reserpine would argue against reserpine being solely responsible for abolition of the C. S. R. in the current study.

Subacute Toxicity.—Six groups of two weanling male albino rats each, weighing 66 to 74 Gm., were fed the basic laboratory diet of a finely ground commercial dog food containing 1861-LLA at levels which would approximate the following daily intake: none (controls), 0.05, 0.5, 5, 25, and 50 mg./Kg., respectively. Each rat was housed individually and food and tap water were offered *ad libitum* throughout the experimental period of twenty-eight days.

TABLE I.—MORTALITY FOLLOWING ORAL INJECTION OF RAUSERFIA, NO. RL-1103 AND NO. 1861-LLA TO RATS

Dose, mg./Kg.	Observation Period, Days						
	1	2	3	4	5	6	7
	RL-1103[a]						
200	0/7	0/7	0/7	0/7	0/7	0/7	0/7
300	1/7	6/7	7/7				
400	4/7	6/7	7/7				
500	7/7						
	1861-LLA[b]						
200	1/7	4/7	6/7	6/7	6/7	6/7	6/7
300	4/7	6/7	6/7	6/7	7/7		
400	6/7	7/7					
500	7/7						

[a] LD_{50}, mg./Kg., 270; confidence limits, 205–356; slope function 1.300.
[b] LD_{50}, mg./Kg., 183; confidence limits, 114–293; slope function, 1.588.

Average body weight, compound consumption, and survival data are presented in Table III. Body weight gains and food consumption were within normal limits at the two lowest dietary levels. Sedation and ptosis were apparent in the 5 mg./Kg./day group during most of the study;

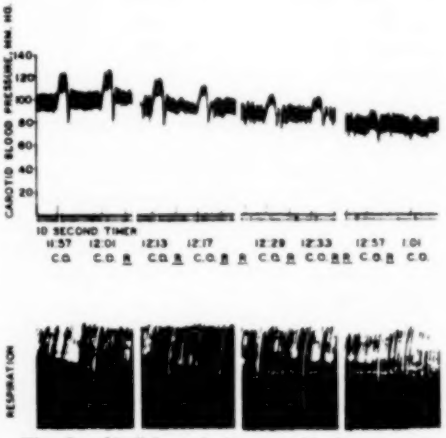

Fig. 2.—Abolition of the carotid sinus pressor reflex by Rauserfia, No. 2290-LLA in the dog. Rauserfia was injected intravenously every four minutes in doses of 10 or 20 μg./Kg. for a total of 190 μg./Kg. in fifty-six minutes. The carotid sinus pressor reflex was standardized initially, then tested two minutes after each dose of Rauserfia. *C.O.* = bilateral carotid occlusion for forty-five seconds; *R* = Rauserfia.

TABLE II.—MORTALITY FOLLOWING INTRAPERITONEAL INJECTION OF RAUSERFIA, NO. RL-1103 AND NO. 1861-LLA TO MICE

Dose, mg./Kg.	Observation Period, Days									
	1	2	3	4	5	6	7	8	9	10
				RL-1103[a]						
10	0/5	0/5	0/5	0/5	1/5	3/5	3/5	3/5	3/5	3/5
46.4	0/5	0/5	0/5	1/5	4/5	4/5	4/5	4/5	4/5	4/5
100	0/5	1/5	1/5	1/5	1/5	2/5	4/5	4/5	4/5	4/5
464	5/5									
1000	5/5									
				1861-LLA[c]						
10	0/5	1/5	1/5	2/5	3/5	4/5	4/5	4/5	4/5	4/5
46.4	0/5	0/5	0/5	1/5	1/5	3/5	5/5			
100	0/5	0/5	1/5	1/5	2/5	3/5	4/5	5/5		
464	1/5	5/5								
1000	5/5									

[a] LD_{50}, mg./Kg., 100–464, [b] 15.0; confidence limits, 5.0–45.0; slope function, 3.9.
[b] By observation at forty-eight hours.
[c] LD_{50}, mg./Kg., 100–464. [b]

TABLE III.—BODY WEIGHTS, COMPOUND CONSUMPTION, AND SURVIVAL DATA FOR MALE ALBINO RATS, TWO ANIMALS PER GROUP, RECEIVING FOR TWENTY-EIGHT DAYS RAUSERFIA, NO. 1861-LLA IN THE DIET AT DOSAGES WHICH APPROXIMATED 0, 0.05, 0.5, 5, 25, AND 50 MG./KG./DAY

Dosage, (mg./Kg./day)	Day No.	Av. Body Wt., Gm.	Av. Body Intake of Food/Rat, Gm.	Av. Daily Intake of 1861-LLA, mg./Kg. Body Wt.	Survival, %
Control	0–7	72–100	13	..	100
	7–14	100–141	18	..	100
	14–21	141–182	17	..	100
	21–28	182–221	20	..	100
0.05	0–7	71–99	13	0.05	100
	7–14	99–127	14	0.04	100
	14–21	127–154	14	0.05	100
	21–28	154–189	16	0.05	100
0.5	0–7	68–106	14	0.58	100
	7–14	106–130	15	0.51	100
	14–21	130–174	16	0.40	100
	21–28	174–210	18	0.52	100
5	0–7	70–73	9	5.4	100
	7–14	73–91	10	5.0	100
	14–21	91–102	8	3.3	100
	21–28	102–131	11	5.9	100
25	0–4	70–54	4	14	100
	4–8	54–41	2	24	0
50	0–2	67–52	2	29	100
	2–5	52–38	0.3	19	0

food consumption and body weight gains were markedly reduced. At the two highest dietary levels there was negligible food consumption after the first day, accompanied by marked loss in body weight. Sedation, ptosis, ataxia, and death occurred in all rats at these dosages. The survivors were sacrificed after twenty-eight days; there were no gross abnormalities of the tissues.

Chronic Toxicity.—Seven groups of weanling male and female albino rats, 10 males and 10 females per group, were selected so that the average body weight in each group was approximately 60 Gm. Each animal was housed individually and fed the basic laboratory diet of a finely ground commercial dog food for an adjustment period of one week. 2290-LLA and RLP-237-268 were then mixed in the diet so that three groups of animals received the former and three groups of animals received the latter in daily dosages which approximated 0.05, 0.5, and 1 mg./Kg., respectively. One group which served as a common control received only the basic diet. After four weeks of feeding, the 1 mg./Kg./day level of 2290-LLA was changed to approximately 2 mg./Kg./day. The food and tap water were offered *ad libitum* throughout the experimental period of ninety days. Weekly records were kept of individual body weight, food consumption, and survival data and are summarized in Table IV.

Ptosis and mild sedation developed as early as the second week in both males and females at levels of 0.5 and 1 mg./Kg./day. There were no other gross pharmacological effects or signs of toxicity. One death occurred after three weeks in the male group receiving 0.5 mg./Kg./day of 2290-LLA while another death occurred after seven and one-half weeks in the male group receiving 1 mg./Kg./day of RLP-237-268. In both instances, death was apparently due to respiratory infection unrelated to ingestion of the compounds. Cell volumes (hema-

TABLE IV.—SUMMARY OF WEIGHTS, COMPOUND CONSUMPTION, AND SURVIVAL DATA FOR MALE AND FEMALE ALBINO RATS RECEIVING FOR A PERIOD OF THIRTEEN WEEKS RAUSERFIA, No. 2290-LLA OR No. RLP-237-268 IN THE DIET AT DOSAGES WHICH APPROXIMATED 0, 0.05, 0.5, AND 1.0 MG./KG./DAY

Dosage, mg./Kg./day	No. Rats in Group	Av. Body Wt. Initial, Gm.	Av. Body Wt. Final, Gm.	Av. Daily Intake of Food/Rat, Gm.	Av. Daily Intake of Compound, mg./Kg. Body Wt.	Survival, %
2290-LLA, Males						
0	10	56	330	14.85	..	100
0.05	10	57	358	16.39	0.05	100
0.5	10	59	348	15.71	0.5	90[b]
1.0[a]	10	58	194	13.93	0.9	100
2.0	10	194	311	13.37	2.0	100
2290-LLA, Females						
0	10	60	210	12.33	..	100
0.05	10	61	208	12.22	0.05	100
0.5	10	55	236	12.72	0.5	100
1.0[a]	10	58	156	12.33	0.9	100
2.0	10	156	227	12.41	1.9	100
RLP-237-268, Males						
0.05	10	55	352	15.97	0.05	100
0.5	10	60	337	15.41	0.5	100
1.0	10	57	329	13.93	1.0	90[c]
RLP-237-268, Females						
0.05	10	65	206	12.10	0.05	100
0.5	10	59	225	13.02	0.5	100
1.0	10	61	227	12.68	1.0	100

[a] Changed to approximately 2.0 mg./Kg./day after four weeks.
[b] One death occurred after three weeks.
[c] One death occurred during the seventh week.

tocrits) were conducted on 5 males and 5 females from each group at termination. The values indicated no incidence of anemia.

Male rats at each dosage of both drugs showed body weight gains which were comparable to the controls. The mean terminal body weights of the 0.05 and 0.5 mg./Kg./day levels of 2290-LLA and of the 0.05 mg./Kg./day level of RLP-237-268 indicate that there was an increase in body weight; however, this apparent increase was found to be insignificant.[3] Food consumption values for the lower dosages of both drugs were slightly elevated, ranging from 0.5 to 1.5 Gm./day more than the controls. Conversely, the high level groups showed food consumption values which were less than the controls (1 to 1.5 Gm./day); furthermore, there was an apparent decrease in body weight at the 2 mg./Kg./day level of 2290-LLA. These latter effects, while insignificant suggest that sedation may have influenced food intake and, consequently, weight gain.

Female rats at the two higher doses of 2290-LLA and at the highest dose of RLP-237-268 showed weight gains which were significantly[3] increased over those of the controls. Food consumption at all levels was comparable. Since this effect occurred with both samples of the drug, it is suggestive that weight gain is a sexual characteristic manifested in females; however, it is probable that had larger groups of animals been used, weight gain would have also been significant with males at the lower dosages.

After ninety days the rats were sacrificed and gross autopsies performed; there appeared to be no significant changes in any group. Samples of liver, kidney, adrenal, intestine, and testes or ovary were removed, prepared for histologic examination, and stained with hematoxylin and eosin. There was no microscopic evidence[4] of abnormality which could be attributed to the drug.

SUMMARY

1. Four samples of Rauserfia, a derivative of *Rauwolfia serpentina*, have been subjected to toxicologic and pharmacodynamic investigation.

2. The acute oral LD_{50}'s of RL-1103 and 1861-LLA in rats, at forty-eight hours, were determined to be 270 and 183 mg./Kg., respectively. There was no significant difference in these values. Following intraperitoneal administration to mice, the LD_{50}'s at forty-eight hours appear to be between 100 and 464 mg./Kg. for both materials. Marked losses in body weight were associated with latent mortality in both rats and mice, suggesting that undernourishment and/or malnutrition were responsible for latent deaths.

3. In the dog, intravenous injection of 2290-LLA caused a pronounced fall in blood pressure and apparent respiratory depression. Furthermore, the material was found to be capable of abolishing the carotid sinus pressor reflex at doses of 190 μg./Kg.

4. Small groups of rats tolerated as much as

[3] $p = 0.05$.

[4] Tissues were examined by Dr. V. J. Dardin.

0.5 mg./Kg./day of 1861-LLA for twenty-eight days with no gross observable effects. At doses of 5 mg./Kg./day there were growth retardation, sedation, ptosis, ataxia, and reduced food consumption. Mortality was obtained in all animals at doses of 25 mg./Kg./day and above.

5. Ninety-day feeding studies in male and female rats indicated significant weight gain in females receiving 0.5 and 2 mg./Kg./day of 2290-LLA and in females receiving 1 mg./Kg./day of RLP-237-268. Ptosis and mild sedation occurred in both females and males at levels of 0.5 mg./Kg./day and above. There was no mortality which could be attributed to the compound; cell volumes indicated no incidence of anemia; and gross or microscopic examination of tissues at sacrifice revealed no significant pathology.

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A Vitamin B_{12} Binding Substance Obtained from *L. Leichmannii* 7830*

By E. M. STAPERT, J. W. GOFF, and L. STUBBERFIELD

A substance which binds vitamin B_{12} was obtained from the *L. Leichmannii* 7830 organism and the U. S. P. medium in which this organism was cultured. This substance inhibits the growth of *L. Leichmannii* 7830. Extracts which were Selas candle filtered, charcoal adsorbed, and freeze dried retained their activity. The dried cell extract was about three times more active in binding vitamin B_{12} than dried used medium.

PRIOR TO the adoption of a standard cleaning technique (1) for culture tubes used in the U. S. P. microbiological assay (2) for vitamin B_{12}, erratic results were obtained when tubes were reused. Acid-cleaned tubes eliminated this irregularity. These erratic results indicated the presence of an inhibitor substance for *L. Leichmannii* 7830 in tubes not acid cleaned. In a continuing study of assay difficulties encountered in the U. S. P. method for vitamin B_{12} it was of interest to determine the source and some of the characteristics of this inhibitor substance.

EXPERIMENTAL

It is evident that the used test medium or the *L. Leichmannii* organism is the source of the inhibitor substance. To verify this we tested the following aseptically prepared materials.

A. Used Medium Centrifuged.—*L. Leichmannii* 7830 was cultured in U. S. P. basal medium to which had been added 0.5 mμg. of vitamin B_{12} per 10 cc. After twenty-four hours incubation, the medium was centrifuged to obtain a solution free of cells. The use of a cell-free solution eliminates the possibility of interference by the cells in determining inhibition. Davis, Layton, and Chow (3) have reported that *L. Leichmannii* cells have the ability to incorporate exogenous vitamin B_{12} from the medium into the cell.

B. Used Medium Centrifuged, Filtered, Charcoal Adsorbed, and Freeze dried.—One liter of a cell-free solution prepared as in A, was filtered through a 0.02 Selas candle. This filtrate was treated with 200 Gm. charcoal (Darco G 60) at *p*H 4.5 for one hour and filtered clear with the aid of filter cell. The final solution was concentrated by freeze drying.

C. Cell Extract, Centrifuged, Filtered, and Charcoal Adsorbed.—Four cc. of packed *L. Leichmannii* 7830 cells was suspended in 50 cc. of distilled water, autoclaved for six minutes at 15 p. s. i., cooled, and centrifuged at 2,500 r. p. m. for twenty minutes. The supernatant was filtered through a 0.02 Selas candle. This solution was treated with 20 Gm. of charcoal (Darco G 60) for twenty minutes and filtered clear with the aid of filter cell.

D. Cell Extract, Centrifuged, Filtered, Charcoal Adsorbed, and Freeze dried.—Same as C except the final solution was concentrated by freeze drying.

* Received August 15, 1955, from the Biological Control Laboratories of The Upjohn Company, Kalamazoo, Mich.

PROCEDURE

These preparations were tested for inhibition of growth by adding them to vitamin B_{12} standards prepared as directed in the U. S. P. (2) microbiological assay. The U. S. P. method was modified by using duplicate dose levels and determining growth turbidimetrically after twenty-four hours' incubation. All culture tubes used were acid cleaned (1). The procedure for adding the test materials to the standards and the results obtained are as follows:

One cc. of solution A used medium was added aseptically to each tube of an autoclaved U. S. P. vitamin B_{12} standard. A second standard was prepared to which solution A was added before autoclaving. The final volume of these experimental standards and a control was 11 cc. per tube. The results in Fig. 1, curves (a) and (b), show there is considerable inhibition of growth of the test organism when solution A is added aseptically. Curve C shows no inhibition of growth when solution A is added before autoclaving.

This inhibition of growth of *L. Leichmannii* by medium in which it had grown for twenty-four hours was unexpected. If the U. S. P. titrimetric procedure had been used the organism would have continued to grow in this medium for an additional forty-eight hours.

It would appear that heat destroys the activity of this inhibiting material. However, this does not explain the inhibition observed (1) in reused tubes washed with water or detergent. Those tubes were subjected to heat during sterilization as required by the assay procedure.

The metabolic requirements of the organism should be satisfied since the standard tubes contained fresh medium. Additional vitamins excluding vitamin B_{12} did not counteract this inhibition.

These results led us to believe that the active material is produced by the *Leichmannii* cell since unused medium does not exhibit inhibition activity. The advantage of using a cell extract would be the elimination of the medium components and some metabolic by-products. In preparing cell extract C the solution was Selas candle filtered and charcoal adsorbed. The method and results of inhibition assays are as follows: A sample of solution C cell extract after Selas candle filtration and prior to charcoal adsorption was tested for inhibition. One cc. of filtrate was added to each tube of an autoclaved standard. A second sample following charcoal adsorption (solution C) was assayed by adding 1 cc. to each tube of two standards. One standard was autoclaved before and the other autoclaved after the addition of the sample. A fourth standard served as a control. All four standards contained a total of 11 cc. per tube. The results are shown in Fig. 2 curves (a), (b), (c), and (d). Curve (b), the solution after Selas candle filtration, shows considerable inhibition when compared with the control curve (a). This is evidence that the cell contains the inhibitor and it can be extracted by the method employed.

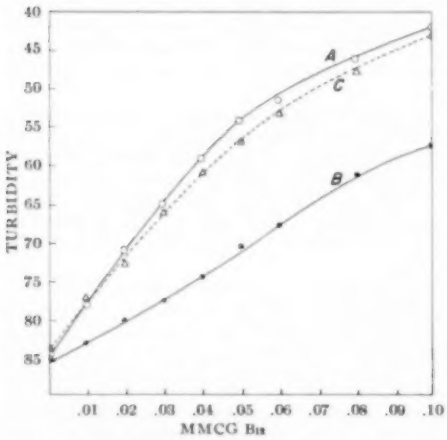

Fig. 1.—Used medium showing inhibition and reversal; *A* ○—control standard; *B* ●—standard with "solution A" used medium added after autoclaving; *C* Δ—standard with "solution A" used medium added before autoclaving.

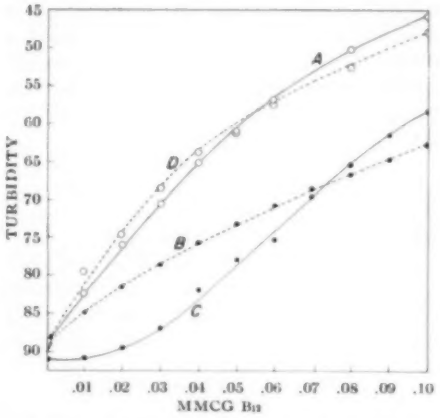

Fig. 2.—Cell extract showing vitamin B_{12} binding and release; *A* ○—control standard; *B* ●—standard with cell extract (Selas filtered) added after autoclaving; *C* ●—standard with "solution C" cell extract added after autoclaving; *D* ○—standard with "solution C" cell extract added before autoclaving.

Bird, *et al.* (4) have reported on the vitamin B_{12} binding power of proteins. They show that several proteins bind vitamin B_{12} when measured by adding them unheated to tubes of assay medium containing vitamin B_{12} and noting the inhibition of growth. Their method of release was to autoclave the protein with the assay medium. Normal growth rates were then restored.

In Fig. 1 and 2 the (b) curves show inhibition of growth. In Fig. 1, curve (c), normal growth was restored when the active material and standard were autoclaved together. This indicates that the active material may bind vitamin B_{12}.

Curves (c) and (d), Fig. 2, show the results of solution C cell extract when added to the medium before and after autoclaving. Curve (c) shows inhibition, whereas curve (d) shows no inhibition with

growth identical to the control standard. Since the experimental conditions and results are comparable to those of Bird we believe this substance to be a vitamin B_{12} binding material.

The charcoal adsorption step following Selas candle filtration removed some growth promoting substance(s) as shown by a comparison of the lower levels of curves (b) and (c). There is no growth in the first levels of curve (c). Comparable results for vitamin B_{12} binding and release were obtained with used medium when it was treated and assayed like solution C.

The vitamin B_{12} binding activity of dried material from used medium B and cell extract D was tested by a method devised by Ternberg and Eakin (5). A vitamin B_{12} solution was added to dried materials B and D and allowed to stand at 25° for two hours. These mixtures were then assayed for unbound vitamin B_{12} by addition to previously sterilized medium. Table I shows the results of this assay.

TABLE I.—BINDING POWER OF DRIED MATERIAL

Material Added, mg.	B_{12} added, µg.	Free B_{12}, µg.	Bound B_{12} %
Used medium B 250	15	10.45	31.3
Cell extract D 70	15	11.35	24.3

These results show the dried cell extract to be approximately three times more potent in binding power than dried used medium. This is further evidence that the *L. Leichmannii* cell produces the binding substance. The cell extract substance showed a positive biuret reaction for proteins. The results of an attempt to dialyze the active material through a Visking cellulose membrane were inconclusive.

SUMMARY

1. A substance which inhibits the growth of *L. Leichmannii* 7830 is present in the medium and in concentrates prepared from the medium in which this organism is cultured.

2. This inhibiting material can be extracted from *L. Leichmannii* cells.

3. Used medium or cell extracts which are Selas candle filtered, charcoal adsorbed, and freeze dried retain their activity.

4. Inhibition of growth caused by the addition of this factor(s) can be prevented by heating in the presence of the medium.

5. We consider this substance to be a vitamin B_{12} binding factor. The dried cell extract is about three times more active in binding vitamin B_{12} than dried used medium.

6. These results may assist in evaluating erratic results encountered in the U. S. P. assay method for vitamin B_{12}.

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Zinc Oxide Hyperfine: Preparation and Properties*

By JOHN B. CONN, W. KARL HUMPHREY, JAMES V. MAGEE, and WILLIAM J. WALLACE†

Zinc Oxide Hyperfine is produced by low-temperature calcination of zinc carbonate ammoniate. It possesses unusual physical properties: the color is brick red, the particle size is about 200 Å, it is hygroscopic, and cements in water. Heating at temperatures exceeding 400° causes it to revert to ordinary zinc oxide. X-ray diffraction evidence indicates disorder in the crystal lattice together with extremely small particle size as being responsible for the observed effects.

IN THE COURSE OF studies into the preparation of finely divided and reactive metal oxides, it was observed that when zinc carbonate ammoniate ($ZnCO_3.NH_3$) is decomposed by heat at temperatures under 400°, the resulting oxide is colored pink to brick red, the coloration being more intense at lower ignition temperature and shorter contact time. Exposure to elevated temperature causes the color to fade irreversibly, the material eventually becoming indistinguishable from ordinary zinc oxide. It was further observed that the solid is quite hygroscopic, and that when moistened with water, it sets to a coherent cake in a few minutes at room temperature. The X-ray diffraction powder pattern is that of zincite, but the lines are

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diffuse, indicating very small particle size, crystal lattice disorder, or both.

Inspection of the literature disclosed that a red zinc oxide had been prepared by Kutzelnigg (1) by heating zinc oxide or nitrate with ammonium nitrate to deflagration. He found that the coloration could be produced only in the presence of ammonia, and concluded that it is a consequence of contamination of nitrogen compounds. About ten years later, Ehret and Greenstone (2) repeated Kutzelnigg's preparation and studied the material in some detail; they associated the color with the existence of a slight excess of zinc in the oxide, which they considered to be present in atomic dispersion as F-centers, rather than as a colloid. The intervention of ammonia they believed to be incidental to the reduction process. The red oxide was found to be highly crystalline.

We prepared some of this red oxide as described by Ehret and Greenstone, and found it to be similar to our material only in color. It is not hygroscopic, shows no cementing property, and confirming the above authors, gives very sharp X-ray diffraction lines. On the other hand, zinc oxide produced by low-temperature calcination of zinc carbonate which contained no ammonia showed X-ray line broadening, but no setting property. Evidently the properties of the new oxide, hereinafter termed Zinc Oxide Hyperfine, are associated both with the state of division and the factor conferring color. Its preparation and characteristics are described in detail below.

PREPARATION

To a solution of 800 Gm. commercial ammonium carbonate in 4 L. 28% ammonia water is added with stirring 880 Gm. zinc oxide U. S. P.; a small amount remains undissolved. The solution is filtered, and carbon dioxide is passed in (preferably under pressure) with stirring, until no further separation of zinc carbonate ammoniate occurs. The washed and dried product weighs about 1300 Gm. (85%).

The zinc carbonate ammoniate is heated at 250° under continuous agitation until ammonia and carbon dioxide cease to be evolved, the weight loss being about 43%. The brick-red, finely divided oxide is stored in tightly closed bottles.

PROPERTIES

Appearance.—*Visual.*—Finely divided, mobile, brick-red powder. When rubbed on the palm, it penetrates the skin, leaving a sticky sensation at the area. Bulk, 0.9 ml./Gm. (tapped).

Microscopic.—In nonaqueous dispersion media (e. g. mineral oil) the oxide particles tend to retain the form of the zinc carbonate ammoniate crystals from which they were derived, showing as prismatic shapes of 30–50 microns size; in aqueous dispersions (1% Darvan No. 1) these pseudomorphs disintegrate into particles 99% of which are in the range 0–5 microns.

Fig. 1.—Aggregate particle of Zinc Oxide Hyperfine × 80,000. Distance between marks at bottom represents 1 μ.

Electron Microscopic.—The fine structure of the above visual shapes is seen in Fig. 1. The crystallites are approximately 200 Å (0.02 μ) in diameter.

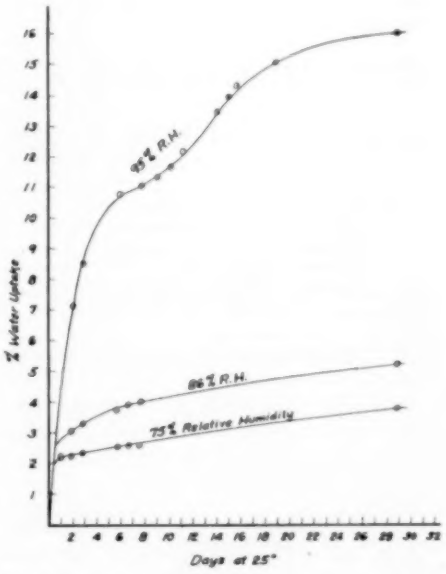

Fig. 2.—Moisture uptake measured over a period of thirty days at fixed relative humidities.

X-ray Diffraction.—Application of the von Laue equation (3) was made to the (10$\bar{1}$2), (11$\bar{2}$0), and (10$\bar{1}$3) reflections, using copper Kα radiation and the Ehret-Greenstone oxide as standard (Norelco wide-range diffraction equipment). From the observed line-broadenings, figures in the range 50-100 Å were obtained for the particle size.

Hygroscopicity.—Moisture uptake was measured over a period of thirty days at fixed relative humidities, with results graphed in Fig. 2. The large increase in hygroscopicity at R. H. greater than 90% is noteworthy. Absorption of water occurs without appreciable caking. Zinc oxide U. S. P. showed no evidence of hygroscopicity under these conditions.

Cementation Effect.—When the powder is kept undisturbed under water at room temperature for twenty-four hours, a firm cake is formed; likewise, a paste prepared from it with water dries to a coherent mass. In neither this case nor the preceding is there any alteration in the X-ray diffraction pattern.

Analysis.—Average run material will contain 90–93% ZnO, 0.1–0.5% ammonia, 0.0–0.2% carbon dioxide, and 7–10% water.

DISCUSSION

The ensemble of properties which distinguish Zinc Oxide Hyperfine from conventional zinc oxides are certainly of physical origin, arising from the extremely fine state of division and the lattice disorder. The observation that X-ray line broadening gives apparent particle sizes which are less than half those shown by the electron microscope is evidence for the extent of disorder, inasmuch as it is superimposed on the effect of reduced particle size. The hygroscopicity and possibly also the cementation effect can be accounted for in the sorption of water in the porous structure left by abstraction of ammonia and carbon dioxide from the zinc carbonate ammoniate crystal. These physical characteristics likewise lead to enhanced chemical reactivity. Copeland and Brauer, for example, have shown that the setting of zinc oxide-eugenol dental cement is a consequence of reaction to form a eugenolate, and that this reaction is very much faster and more extensive when Zinc Oxide Hyperfine is used (4). It therefore offers advantages in topical formulations which employ zinc oxide.

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Determination of Hexachlorophene in Liquid Soaps*

By RICHARD F. CHILDS† and LLOYD M. PARKS

A differential spectrophotometric method of assay for hexachlorophene in liquid soaps has been developed. Measurement of the absorbance, at 312 millimicrons, of a dilution of the sample at *p*H 8 in the solute cell *vs.* the same dilution at *p*H 3 in the solvent cell of the spectrophotometer eliminates irrelevant absorption due to the soap base and permits assay of the sample without removal of the soap. The method is proposed for assay of the U. S. P. XV hexachlorophene liquid soap.

WITH THE INTRODUCTION of hexachlorophene liquid soap into U. S. P. XV (1) it is desirable to have an assay for the hexachlorophene content of this preparation that is as convenient, rapid and accurate as possible for U. S. P. purposes. It was the purpose of this study to develop such a procedure and to check its use with several commercial samples of hexachlorophene-containing liquid soaps.

Methods described in the literature for the determination of hexachlorophene in soaps all employ colorimetric or spectrophotometric techniques. The method of Gottlieb and Marsh (2), based on the colorimetric determination of the 4-aminoantipyrine-phenol-potassium ferricyanide complex, is reported to give unreliable results (3, 4). The method of Larson (3), based on the colorimetric determination of the phenol-ferric chloride salt, a modification of which has been adopted by the U. S. P. XV (1), involves precipitation of the soap with barium bromide. In our hands the fleeting color produced in this method made it difficult to obtain reproducible results. The rapid spectrophotometric method of Lord, *et al.* (4), is applied directly to the soap sample and involves measurement of its extinction coefficient in 0.1 *N* potassium hydroxide solution at three different wavelengths, in the region of the peak absorption of hexachlorophene;

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a correction for irrelevant absorption due to the soap base is made by application of the Morton-Stubbs three-point geometrical correction. The spectrophotometric procedure of Clements and Newburger (5) is designed for the determination of hexachlorophene in all types of cosmetic formulations and involves several extractions which would appear to be unnecessary for the determination of its content in a liquid soap.

EXPERIMENTAL

Preliminary Experiments.—Since hexachlorophene can be quantitatively titrated with standard alkali and also has a characteristic absorption peak at 298 mμ (5) preliminary attempts were made to determine the hexachlorophene in a sample, after removal of the soap, by titration and by spectrophotometric means. The soaps were precipitated from samples of liquid soap bases containing known amounts of hexachlorophene by the addition of aqueous barium chloride, the filtrate acidified with diluted hydrochloric acid, and the hexachlorophene extracted with successive portions of ether. Titration of the ether residues gave high results, indicating the presence of fatty acids which had escaped barium precipitation. Du Claux values on these residues indicated the acids to be two to four carbons in length. Absorbances of the ether residues in 80% methanol at 298 mμ also gave high results, with samples containing hexachlorophene as well as with the soap bases alone. This substantiated the irrelevant absorption due to the soap base that was noted by Lord, *et al.* (4).

The Differential Spectrophotometric Method.—Since it is known that a substance of equal concentration in both the solute and solvent cells of the spectrophotometer gives an absorption that is essentially the same as a cell blank curve, it appeared that such a technique might eliminate the error caused by absorption of the soap base by allowing the instrument to cancel out the irrelevant absorption. This was demonstrated experimentally by determining the absorbances of samples of soap bases in that manner.

Alkali increases the intensity of absorption of phenols, by converting them from the molecular to the ion or salt form, and also shifts their absorption peaks to longer wavelengths (6, 7). To determine the *p*H at which hexachlorophene exists entirely in the ion form the absorbances of solutions of 5 mg./100 cc. of the compound in 90% methanol at various *p*H values, in the solute cell, *vs.* 90% methanol in the solvent cell were determined. From these data it was apparent that below *p*H 4.0 hexachlorophene exists essentially in the molecular form and above *p*H 10.0 it exists essentially in the ion form. Although the compound showed a slightly greater absorption intensity at *p*H 10.0 than at *p*H 8.0, and thus probably exists in the ion form to a slightly greater extent at the higher *p*H, it was found expedient in the later studies to use a *p*H of approximately 8 when determining the absorption of hexachlorophene in the soap dilutions in order to avoid the development of turbidity. For practical purposes the absorption intensity above *p*H 8 was not sufficiently *p*H-sensitive to introduce a significant error.

Since it had been shown that the irrelevant absorption due to the soap base could be effectively blanked out it was thought that, by determining the absorption of hexachlorophene in alkaline media in the solute cell against its absorption in acid media in the solvent cell, there would be obtained an absorption curve that is unique for the phenol and without interference from the soap base. To test this possibility the absorbances of 5 mg./100 cc. of hexachlorophene in 90% methanol at various *p*H values, in the solute cell, were determined with the same solutions at *p*H 3.0 in the solvent cell, with the results shown in Fig. 1.

The technique, described above, of determining the absorbance of a dilution of hexachlorophene at an alkaline *p*H in the solute cell against its same concentration at an acid *p*H in the solvent cell will be referred to hereafter as the differential spectrophotometric method. By use of this technique it appeared possible to eliminate the irrelevant absorption inherent in the soap bases and also to determine the hexachlorophene content of a liquid soap sample without removal of the soap.

Applicability of the method was checked on a number of commercial soap bases which contained no hexachlorophene. The general procedure used was to dilute a weighed sample of the soap base with 90% methanol, place one aliquot, adjusted to *p*H 8.0, in the solute cell and determine the absorbance with another aliquot of the dilution, adjusted to *p*H 3.0, in the solvent cell. The results shown in Fig. 2 were obtained. From these data it was apparent that the soap bases themselves would not interfere with application of the differential method.

The shift in the wavelength of peak absorption of soap dilutions containing hexachlorophene with a change in *p*H, caused by a change in the compound from its molecular form (in acid) to its ion form (in alkali), along with the sharpness of the curves made possible a "differential" curve of considerable absorption intensity as shown by Curve B in Fig. 3. It was shown that the height of this differential curve represented the distance, perpendicular to the base line, (dotted line) between the absorption of

TABLE I.—DIFFERENTIAL ABSORPTION OF HEXACHLOROPHENE IN 90% METHANOL AT 312 mμ

Concentration, mg./100 cc.	Differential Absorbance (*p*H 8.0 *vs.* *p*H 3.0)	Arithmetic Average
2.00	0.284	0.285
	0.285	
	0.286	
3.00	0.416	0.429
	0.437	
	0.435	
5.00	0.712	0.713
	0.718	
	0.703	
	0.718	
	0.719	
	0.707	
7.00	0.997	0.999
	1.000	

hexachlorophene in its molecular and ionic forms at any wavelength.

Preparation of a Standard Curve by the Differential Method.—The maximum absorption, i. e., the wavelength of peak absorption for the differential curve for hexachlorophene, was determined by measuring the differential absorbance (*p*H 8.0 *vs.* *p*H 3.0) of its solution (2.5 mg. in 50 cc. of 80% methanol) at various wavelengths. Maximum absorption was obtained at 312 mμ.

Solutions of hexachlorophene in 80% methanol, varying in concentration from 1.0 mg./100 cc. to 7.5 mg./100 cc. were shown to obey Beer's law when determined by the differential method (*p*H 8.0 *vs.* *p*H 3.0) at 312 mμ. To obtain a standard curve the absorbances of solutions of hexachlorophene of known concentration in 90% methanol were determined by the differential method (*p*H 8.0 *vs.* *p*H 3.0) at 312 mμ with the results shown in Table I and Fig. 4.

Spectrophotometric Assay for Hexachlorophene in Liquid Soaps

Apparatus and Reagents.—A Beckmann Model DU Quartz Spectrophotometer with matched cells was used. Absolute methanol, acetone free, was used to make the following reagents:

90% methanol (900 ml. of absolute methanol and 100 ml. of water).

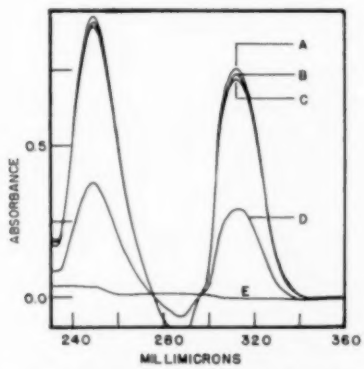

Fig. 1.—Absorbance of hexachlorophene in 90% methanol at varying *p*H (solute cell) *vs.* same concentration at *p*H 3.0 (solvent cell). *A* = *p*H 10.0; *B* = *p*H 9.0; *C* = *p*H 8.0; *D* = *p*H 6.0; *E* = *p*H 4.0, 3.0 and 2.0.

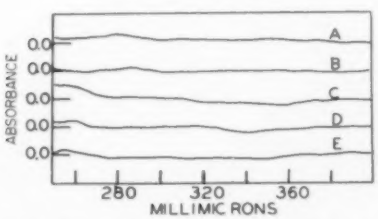

Fig. 2.—Differential absorbance (*p*H 8.0 *vs.* *p*H 3.0) of five different soap bases in 90% methanol.

TABLE II.—HEXACHLOROPHENE ASSAY OF SURGICAL SOAPS

Sample	Hexachlorophene labeled, %	Found, %
Soap base A	0	0.019; 0.017
Soap base A plus 1% added[a]	1.00	1.00; 0.988
Soap base A plus 2% added[a]	2.00	2.03
Soap base B	0	0.008; 0.008
Soap base B plus 1% added[a]	1.00	1.005
Soap base C	0	0.086; 0.06
Soap base D	0	0.004; 0.021
Soap base E	0	0.017
Surg. soap A	1.00	1.051; 1.055
Surg. soap B	1.00	1.02
Surg. soap C	2.00[b]	0.625; 0.627
Surg. soap C plus 1% added[a]	....	1.629
Surg. soap D	0.72	0.76; 0.79
Surg. soap E	0.75	0.77; 0.78
Surg. soap E plus 0.5% added[a]	1.25	1.27; 1.27
Surg. soap F	0.63	0.38; 0.38
Toilet soap G	Unknown	0.30; 0.29
Surg. soap H	1.00	0.97; 0.98

[a] Hexachlorophene added to the 15-ml. aliquot of the final dilution of the sample.

[b] Based on anhydrous soap content; all others on total weight basis.

90% methanol adjusted to *p*H 8.0 with 0.1 *N* NaOH.

0.3 *M* acetic acid solution, prepared by diluting 18 Gm. of glacial acetic acid to 1,000 ml. with 90% methanol.

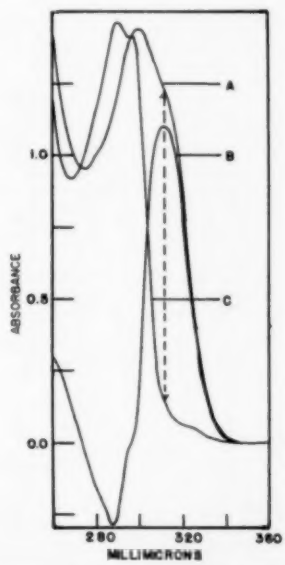

Fig. 3.—Absorbance of a dilution of Soap base B plus 2% added hexachlorophene in 90% methanol. *A* = *p*H 8.4 (solute cell) *vs.* 90% methanol; *C* = *p*H 3.4 (solute cell) *vs.* 90% methanol; *B* = *p*H 8.4 (solute cell) *vs.* *p*H 3.3 (solvent cell).

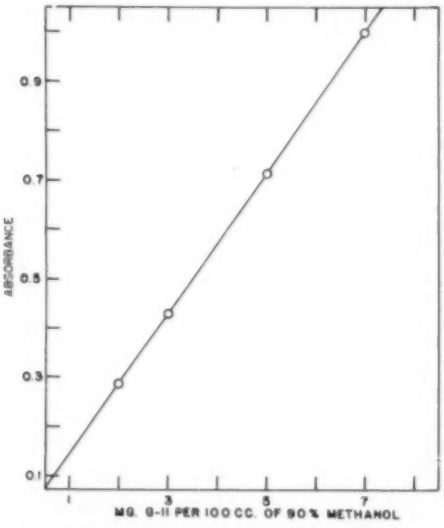

Fig. 4.—Concentration of hexachlorophene *vs.* differential absorbance (*p*H 8.0 *vs.* *p*H 3.0) at 312 mμ.

methanol containing 0.1% by volume of concentrated HCl.

Standard Hexachlorophene Graph.—Dissolve 0.500 Gm. of U. S. P. hexachlorophene, previously dried at 105° for 4 hours, in alcohol to make exactly 100 ml. Dilute a 10-ml. aliquot of this solution to 100 ml. in a volumetric flask with 90% methanol (Solution A). To each of six 50-ml. volumetric flasks add 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 ml. of Solution A leaving the seventh flask as a blank. Fill each of the flasks to volume with 0.3 *M* acetic acid solution and mix thoroughly. This gives a series of solutions containing 0.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mg. of hexachlorophene per 100 ml., respectively, at approximately *p*H 3.

Prepare a second series of standard hexachlorophene solutions, of the same concentration as above, at *p*H 8, as follows: To each of six 50-ml. beakers add 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 ml. of Solution A leaving the seventh beaker as a blank. Add to each beaker approximately 20 ml. of the 90% methanol adjusted to *p*H 8 and add sufficient sodium hydroxide solution to each to bring it to *p*H 8 on the *p*H meter. Wash the electrodes with the 90% methanol adjusted to *p*H 8, transfer quantitatively the contents of each beaker to a 50-ml. volumetric flask, dilute to volume with the 90% methanol adjusted to *p*H 8, and mix thoroughly.

Arrange the two series of solutions in pairs of the same concentration. Place the *p*H 3 member of each pair in the solvent cell of the spectrophotometer, the *p*H 8 member in the solute cell, and plot the observed absorbance at 312 mμ on the ordinate scale against the corresponding concentration of hexachlorophene in mg./100 ml. on the abscissa scale to give a straight line passing through the origin.

Procedure.—Weigh accurately in a weighing bottle about 5 Gm. of the hexachlorophene liquid soap and transfer the sample quantitatively to a 100-ml. volumetric flask. (The transfer is facilitated by using a 7-cm. funnel and using alcohol in a plastic squeeze bottle for washing.) Dilute to volume with alcohol and mix thoroughly. Dilute a 25-ml. aliquot of this solution to 100 ml. in a volumetric flask with 90% methanol and mix thoroughly. To each of two 50-ml. volumetric flasks add 15 ml. of the dilution. Fill one of the flasks to volume with 0.3 *M* acetic acid solution and the other with 90% methanol. This gives a pair of soap dilutions one member of which is at approximately *p*H 3, the other of which is approximately *p*H 8.[1] Place the *p*H 3 member in the solvent cell of the spectrophotometer, the *p*H 8 member in the solute cell, and, from the absorbance observed at 312 mμ and the use of the standard hexachlorophene graph, calculate the mg. of hexachlorophene in 100 Gm. of sample.

Analyses of Samples.—The method was used for the assay of samples of hexachlorophene-containing liquid soaps and the corresponding soap bases, obtained from various manufacturers, with the results shown in Table II.

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[1] All samples assayed in this study showed a *p*H greater than 7.8 when diluted with 90% methanol. If the dilution is below *p*H 7.8 at this point it should be adjusted to *p*H 8 by addition of alkali.

Syntheses of Amino Ethers with Potential Pharmacological Activity*

By CHARLES RIFFKIN and NATHAN RUBIN

A number of potentially active modifications of the basic amino-ether structure R—O—CH_2CH_2—N—R′R″ (when R is alkyl or aryl) were prepared in a study of structure-histaminolytic activity correlation. Although the R group was considered the principal determinant of activity and subject to the most modifications, variation in the amine moiety and elsewhere were also made. Water-soluble salts of the new bases were also prepared in the usual manner.

Considerable evidence has accumulated to demonstrate the nonspecific character of the amino-ether antihistamines. Antispasmodic activity (1), side reactions (2, 3), barbiturate potentiation (4), cerebral effects (5), cardiac (6) and blood pressure changes (7, 8, 9), intestinal motility effects (10), DFP antagonism (11), and an effect on the uterus (13) are but some of the many reported pharmacological responses. Confirmation of cerebral activity was found by Churchill and Gammon (12) who observed that in patients suffering from petit mal there occurred a reduction in frequency of attacks when Benadryl® was administered. It is thus quite apparent that the amino-ether antihistamines are nonspecific in their activity and that in addition to possessing histaminolytic activity they are pharmacologically active on a large number of other organs in the body. Such being the case, it became desirable to ascertain what in the molecular structure of these compounds is responsible for their diverse activity and, if possible, by producing modifications of this structure, to correlate pharmacological activity to molecular make-up.

It was therefore the purpose of the present work to synthesize a number of new compounds of the basic amino-ether structure in order that they may be screened and evaluated for histaminolytic-structural relationships.

DISCUSSION AND METHODS

An examination of the literature reveals that while there are only a limited number of antihistamines of practical value that contain the amino-ether construction as an integral part of the molecule, there have been synthesized and tested a very large number of substances that fall into this type of chemical classification (14–17).

By suitably varying the R, R′, and R″ of the general formula R—O—C—C—R′R″, we were able to obtain the desired modifications of the basic amino-ether structure. Table I shows the range of R groups employed illustrating the alkyl, cycloalkyl, aryl, and aralkyl types. The amine moieties were varied to a lesser extent.

The three general synthetic approaches consisted of: Method A, reaction of the sodium or potassium salt of a hydroxy compound with a dialkylaminoalkyl halide; Method B, reaction of an alkyl halide with the sodium or potassium salt of an aminoalcohol; Method C, aminolysis of a halogen-substituted ether. Isolation of the products was in the usual manner. Hydrochloride salts were prepared in most cases and only when they proved to be hygroscopic were the dihydrogen citrates prepared.

Table I lists the compounds prepared, together with the physical constants and other pertinent data. There were a number of instances where the required intermediates were not available and had to be prepared. Dicyclohexylcarbinol was prepared from cyclohexyl chloride and ethyl formate according to the method of Sabatier and Mailhe (18), while dibenzylcarbinol was prepared by the aluminum isopropoxide reduction of dibenzyl ketone. 2-Benzylcyclohexanol was prepared via 2-chlorocyclohexanol which was converted to cyclohexene oxide (19) which in turn was reacted with benzyl chloride in a Grignard reaction (20) to produce the desired intermediate.

β-Chloroethylbenzhydryl ether was prepared by refluxing an excess of ethylene chlorohydrin with benzhydrol in the presence of a small quantity of concentrated hydrochloric acid (21). Similarly prepared were the γ-chloropropylbenzhydryl ether and the 2-chloroisopropylbenzhydryl ether.

The monocarbethoxy-substituted piperazine was prepared by a modification of the method of Moore, *et al.* (22). This consisted of using a line operated Beckman *p*H meter and maintaining an optimum *p*H of 3 during the addition. This procedure proved more convenient than the original colorimetric method. The monocarbethoxy derivative was in turn methylated by the method of Clarke, *et al.* (23),

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TABLE I.—PROPERTIES OF PREPARED COMPOUNDS

Compound	No.	Method of Prep.	B. p.,[a] °C.	M. p.[b] Salt, °C.	n_D^{22} Base	Analysis[c] Base Calc.	Base Found	Salt Calc.	Salt Found	Yield of Base, %
$C_6H_5CH_2OCH_2CH_2N(CH_3)_2$	1	B	102–3/4 mm.	101–2	1.4995	7.81	7.53	6.46	6.47	63
$(C_6H_5OCH_2)_2CHOCH_2CH_2N(CH_3)_2$	2	A	212–4/4 mm.	127–8	1.5360	4.44	4.45	3.98	3.86	63
$(C_6H_{11})_2CHOCH_2CH_2N(CH_3)_2$	3	A	154–5/5 mm.	173–4	1.4806	5.23	5.32	4.61	4.66	45
p-$C_6H_5OC_6H_4OCH_2CH_2N(CH_3)_2$	4	B	185–8/11 mm.	163–4	1.5884	5.44	5.21	4.76	4.75	66
o-$C_6H_5C_6H_{10}OCH_2CH_2N(C_2H_5)_2$	5	A	170–8/8 mm.	203–4	1.5072	6.50	6.80	5.56	5.85	80
$(C_6H_5)_2CHOCH_2CH_2OCH_2CH_2N(CH_3)_2$	6	B	190–9/5 mm.	130–1	1.5600	4.68	4.61	4.14	4.10	63
$(C_6H_5)_2CHOCHCH_2N$ (O, CH_3)	7	C	173–6/2 mm.	145–6	1.5570	4.49	4.47	4.02	4.05	75
$(C_6H_5)_2CHOCH_2CH_2N$ (N, $NCH_2CH_2OCH(C_6H_5)_2$)	8	B	m.p., 96	170–1	...	5.51	5.39	4.83	4.84	36
$(CH_3)_2CH$ / $CHOCH_2CH_2N(CH_3)_2$ / $(CH_3)_2CH$	9	A	98–9/32 mm.	126–8	1.4277	7.42	7.48	3.69	3.70	89
O, $CHOCH_2CH_2N(CH_3)_2$	10	A	190–9/5 mm.	Hygroscopic	...	5.20	5.17	..	..	10
$(C_6H_5)_2CHOCH_2CH_2N$ (S)	11	B	m. p., 89–90	...	...	3.42	3.43	..	..	25
$C_6H_5CH_2O$, OCH_2C (N—CH_2, $NHCH_2$)	12	A	m. p., 126–7	177–9	...	9.93	9.93	8.78	8.64	27 (HCl)
$(C_6H_5CH_2)_2CHOCH_2CH_2N(CH_3)_2$	13	A	185–6/6 mm.	110–1	1.5360	4.94	5.07	4.38	4.42	48
$(C_6H_5)_2CHOCH$ ($CH_2N(CH_3)_2$, $CH_2N(CH_3)_2$)	14	B	148–50/1 mm.	213–4	1.5437	8.96	8.80	7.27	7.17	35
o-$C_6H_5CH_2C_6H_{10}OCH_2CH_2N(CH_3)_2$	15	A	163–4/5 mm.	86–7	1.5080	5.35	5.48	4.70	4.89	62
o-$C_6H_5CH_2C_6H_4OCH_2CH_2N$ (N, NCH_3)	16	C	200–1/3 mm.	211–2	1.5633	9.39	9.34	7.54	7.64	40
$(C_6H_5)_2CHOCH_2CH_2CH_2N$ (N, $NCH_2CH_2CH_2OCH(C_6H_5)_2$)	17	C	m. p., 91–2	202–4	...	5.24	5.19	4.61	4.55	10

	18	A	183–5/5 mm.	138–9[f]	1.4950	4.77	4.73	2.88	2.86	83	
	19	A	...	143–5[f]	...	...	..	5.93	5.85	10[g]	
	20	C	173–5[f]/12 mm.	154–5[f]	...	...	..	4.03	4.14	10	
	21	A	139–40/3 mm.	93–4[f]	1.4988	5.56	5.37	3.15	3.10	33	

[a] Uncorrected. [b] Determined with a modified Thiele melting point apparatus and short scale thermometers. The salts are hydrochlorides.
[c] Kjeldahl nitrogen determinations. Additional confirmation was obtained by nonaqueous titrations wherein the results were in close agreement.
[d] Extremely hygroscopic. Melting point done in sealed tube; material for analysis stored and dried over P_2O_5.
[e] The free base was a glass-like material which converted into the citrate. [f] These salts are the citrates. [g] Isolated as the citrate.

and subsequently hydrolyzed by concentrated hydrochloric acid to give N-methylpiperazine dihydrochloride monohydrate, which was in turn reacted with the proper chloroether after neutralization and removal of the water by distillation with xylene.

The symmetrically substituted piperazines were prepared by reacting two to one molar proportions of the proper chloroether with anhydrous piperazine in dry toluene in the presence of a catalytic amount of copper powder and an acid acceptor in the form of anhydrous sodium carbonate. To obtain the anhydrous piperazine, a corresponding quantity of the hexahydrate was added to an excess of toluene and the water distilled off by azeotropic distillation.

For the preparation of the imidazolines, the method of Wheatley, *et al.* (24), was unsatisfactory and the best results were obtained by preforming the halomethylimidazoline hydrochloride obtained by the method of Klarer and Urech (25), liberating the free halogenated base with concentrated alkali and subsequently condensing it with the proper alkali metal derivative. By carrying out the final condensation in a nonpolar medium, excessive polymerization was avoided.

The results of the pharmacological studies will be published at a later date.

EXPERIMENTAL

Method A.—*β-Dimethylaminoethyl-1,3-di-(phenoxy)-isopropyl ether,* (compound 2).—In a 1-liter, 3-necked flask fitted with a reflux condenser and drying tube, mechanical stirrer, and dropping funnel, was placed 5 Gm. of a 10-micron dispersion of metallic sodium,[1] equivalent to 2.5 Gm. of sodium and 200 cc. of dry xylene. Twenty-five grams of 1,3-di-(phenoxy)-2-propanol dissolved in 100 cc. of dry xylene was added slowly to form the sodium derivative. In preparation for this compound, 20 Gm. of dimethylaminoethyl chloride hydrochloride was taken the day before and dissolved in a minimum of water to which was added 50 cc. of xylene, followed by an excess of solid sodium hydroxide. The mixture was shaken mechanically for two hours whereupon the free base dissolved in the xylene layer. This layer was then separated, dried over anhydrous calcium chloride, and placed in a freezer overnight. The solution was suitable for use in this form and as such was added to the above sodium derivative in dry xylene with refluxing and stirring continued for six hours. After allowing the mixture to cool, dilute aqueous alkali was added to dissolve the inorganic salt, the desired product remaining in the organic phase. The free base was then extracted from the organic phase by means of repeated extractions with dilute hydrochloric acid. Addition of solid potassium carbonate liberated the free base which was taken up in ether, dried, and distilled. See Table I for physical constants and analytical data.

Method B.—*β-Dimethylaminoethoxyethyl benzhydryl ether* (compound 6).—In a 500-ml. 3-necked flask fitted with a reflux condenser and drying tube, mechanical stirrer, and dropping funnel, was placed 4.6 Gm. of a 10-micron dispersion of metallic sodium,

[1] Supplied by National Distillers Corporation, New York City.

equivalent to 2.3 Gm. of sodium, and 100 cc. of dry xylene. Nine grams of dimethylaminoethanol in 50 cc. of dry xylene was added dropwise and the mixture stirred until the formation of the sodium derivative was complete (approximately two hours). Twenty-five grams of β-benzhydryloxyethyl chloride in 50 cc. of dry xylene was then added and the mixture refluxed and stirred for six hours whereupon the precipitation of the inorganic sodium chloride appeared to be complete. The mixture was cooled and worked up in the usual manner. See Table I for physical constants and analytical data.

Method C.—*N-(o-Benzylphenoxyethyl)-N'-methyl piperazine* (compound 16).—*o*-Benzylphenol was prepared according to Kremers, *et al.* (26), and then alkylated by the Clemo and Perkins procedure (27) to yield the β-chloroethyl ether of *o*-benzylphenol melting at 65–66°. Thirty grams of this material was mixed with 32 Gm. of N-methylpiperazine dihydrochloride monohydrate, 30 Gm. of anhydrous sodium carbonate, and 2 Gm. of copper powder suspended in 200 cc. of dry xylene. This mixture was distilled until the thermometer read 130°. The condenser was then set for reflux and the mixture was stirred and refluxed for eighteen hours. After cooling, water was added and the reaction mixture worked up in the usual manner. See Table I for physical constants and analytical data.

SUMMARY

Twenty-one new compounds and their salts have been prepared. All have in common an amino-ether construction and therefore, pharmacologically, should resemble known antihistaminic agents. The variations, moreover, present an opportunity for correlating structure to activity and enable some conclusions to be drawn with reference to the side effects produced as well.

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Antimicrobial Properties of Dyclonine Hydrochloride, A New Topical Anesthetic*

By H. J. FLORESTANO and M. E. BAHLER

Dyclonine hydrochloride (4-*n*-butoxy-β-piperidinopropiophenone hydrochloride) has been found to possess, in addition to its topical anesthetic properties, significant bactericidal and fungicidal activity. Self-sterilizing action manifested by preparations containing the drug was considerably enhanced upon the addition of chlorobutanol. Results of *in vitro* tests employing microorganisms commonly involved in local infections indicated that the two agents in combination act synergistically.

THERE APPEARS to have been very little attention given in the past to study of the antimicrobial properties of agents intended for topical anesthesia. That such concomitant action would be of definite advantage is apparent, not only from the standpoint of prevention and control of infection but for formulation purposes as well.

Dyclonine hydrochloride (4-*n*-butoxy-β-piperidinopropiophenone hydrochloride) is a white, crystalline powder possessing topical anesthetic properties characterized by rapid onset of effect, lack of systemic toxicity, and a low index of sensitization (1–7). In addition to its effective-

* Received November 2, 1955, from the Research Department, Pitman-Moore Company, Division of Allied Laboratories, Inc., Indianapolis, Ind.

ness and wide margin of safety, the compound has been found to have significant bactericidal and fungicidal activity, details of which are presented in this report. Of further interest was the observation that combinations of dyclonine hydrochloride with chlorobutanol exerted a synergistic action on a number of microorganisms frequently involved in skin and mucous membrane infections.

EXPERIMENTAL

The *in vitro* activity of dyclonine hydrochloride was tested by the serial twofold dilution method against the following organisms: *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Candida albicans, Microsporum lanosum,* and *Trichophyton mentagrophytes*. The synthetic broth described by Sahyun, *et al.* (8), was employed as the test medium for *Escherichia coli*, and that of Straus, *et al.* (9), used for *Staphylococcus aureus*. The remainder of the bacterial species were tested in brain heart infusion broth (Difco), while Sabouraud maltose broth served for the yeast and molds. In most instances comparison of activity was made with that of chlorobutanol[1] and phenol. Stock solutions of dyclonine hydrochloride were prepared in distilled water, which was adjusted to *p*H 4.0 with 0.1 *N* HCl for maintenance of stability. Stock solutions of chlorobutanol and phenol were made up in the respective media. All initial drug solutions were sterilized by passage through sintered glass filters. Final drug dilutions were contained in a volume of 5 cc. for the bacterial tests and in 10 cc. for the yeast and molds. The various inocula introduced per cc. of drug broth are given in Table I. Tubes inoculated with bacteria and the yeast were incubated at 37° and examined at twenty-four-hour intervals. Those showing no growth at the end of twenty-four and forty-eight hours, respectively, were subcultured into fresh sterile broth for determination of the minimum lethal concentrations for each organism. Mold tests were incubated at 28° with subcultures performed at seven and fourteen days for fungicidal end points.

Results are summarized in Table I. Since the microbicidal concentrations of each of the three agents coincided usually with the apparent static end points (minimum concentrations preventing visible growth), data shown are in terms of minimum lethal concentrations. *Staphylococcus aureus* was found to be the most susceptible of the test organisms to dyclonine hydrochloride, while *Pseudomonas aeruginosa* proved the most resistant. With the exception of the latter organism, the action of dyclonine hydrochloride was greater than that of chlorobutanol or phenol for the species compared. Ten per cent normal horse serum incorporated in the medium did not appear to alter significantly the *in vitro* effectiveness of dyclonine hydrochloride.

Phenol Coefficient.—Employing the F. D. A. phenol coefficient method for testing antiseptics (10), the greatest dilution of dyclonine hydrochloride killing *Staphylococcus aureus* in ten minutes was 1:325, or a concentration of 0.31% (Table II). In comparison, a 1:90 dilution of phenol, or 1.11%, was required to produce the same effect, while a concentration of greater than 0.5% was indicated for chlorobutanol. Limitations of solubility precluded the testing of chlorobutanol at less than a 1:200 dilution. As indicated in Table II, the absolute phenol coefficient of dyclonine hydrochloride in plain aqueous solution (*p*H 4.2) was 3.6. When chlorobutanol in a concentration of 0.33% was added to a 0.5% isotonic aqueous solution of dyclonine hydrochloride, the phenol coefficient of the mixture was greater than with either agent alone, i. e., 4.4.

The finding that dyclonine hydrochloride is a stronger bactericide than phenol appears in agreement with published data (Table III). The possibility that dyclonine hydrochloride and chlorobutanol in combination may act synergistically was suggested by the increase in phenol coefficient following mixture of the two agents.

Synergism Studies.—Two different methods were used in testing for possible synergistic action between dyclonine hydrochloride and chlorobutanol. One entailed the comparison of minimum lethal concentrations of each agent separately and in combination by the tube dilution procedure. The second method involved the determination of differences in killing rates by interval colony counts when the drugs were used alone and together.

Studies by the tube dilution technique were made with the following organisms: *Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa,* and *Candida albicans*. Brain heart infusion broth was employed for the bacterial species and Sabouraud maltose broth for the yeast. Based on previously determined minimum microbicidal concentrations of dyclonine hydrochloride and chlorobutanol singly, combinations of various fractional amounts of these end points were prepared in 5 cc. of broth. Inocula consisted of 0.1 cc. of an eighteen-hour culture of test organism diluted so that the number of cells for the various species ranged from 4,500 to 19,000 per cc. of final tube. Tests were incubated at 37°. Tubes showing no visible growth at twenty-four and forty-eight hours were subcultured into fresh broth to ascertain minimum lethal concentrations.

TABLE I.—COMPARATIVE ANTIMICROBIAL ACTIVITY OF DYCLONINE HYDROCHLORIDE, CHLOROBUTANOL, AND PHENOL

Organism	Inoculum per cc.	Minimum Microbicidal Concentration, %: Dyclonine HCl	Chlorobutanol	Phenol
Staphylococcus aureus	1,900	0.006	0.4	0.4
Bacillus subtilis	20	0.025	>0.4	0.4
Escherichia coli	1,200	0.012	0.2	0.2
Proteus vulgaris	19,000	0.05	0.2	0.2
Pseudomonas aeruginosa	15,000	0.8	0.4	0.2
Candida albicans	1,900	0.2	>0.4	0.4
Microsporum lanosum	39,000	0.1	..	..
Trichophyton mentagrophytes	147,000	0.025	..	..

[1] Concentrations given for chlorobutanol throughout the report are in terms of the anhydrous form.

TABLE II.—HIGHEST DILUTION OF DYCLONINE HYDROCHLORIDE, CHLOROBUTANOL, AND PHENOL KILLING STAPHYLOCOCCUS AUREUS AFTER TEN MINUTES BUT NOT AFTER FIVE MINUTES AT 37°

Agent	Dilution	Concn., %	Phenol Coefficient
Dyclonine HCl	1:325	0.31	3.6
Chlorobutanol	<1:200	>0.5	<2.2
Phenol	1:90	1.11	1.0
Dyclonine HCl + Chlorobutanol	1:400 + 1:667	0.25 + 0.15	4.4

TABLE III.—ANTIBACTERIAL ACTIVITY OF DYCLONINE HYDROCHLORIDE[a]

	Bactericidal Action: Rideal-Walker Phenol Coefficient Method			
	Staphylococcus aureus		*Escherichia coli*	
Agent	Concn. Tested, %	Killing Time, Minutes	Concn. Tested, %	Killing Time, Minutes
Dyclonine HCl	0.3	10	0.2	120
Phenol	0.8	30	0.8	30

[a] Data from Profft, E., *Chem. Tech. (Berlin)*, **5**, 13(1953).

TABLE IV.—SYNERGISTIC ACTION OF DYCLONINE HYDROCHLORIDE AND CHLOROBUTANOL

	Lethal Combinations in Terms of Fractional Portions of the Minimum Microbicidal Concentrations of Each Agent When Used Alone[a] Dyclonine HCl + Chlorobutanol	
Organism	24 Hr.	48 Hr.
Staphylococcus aureus	1/2 + 1/2	1/2 + 1/4
Proteus vulgaris	1/2 + 1/4	1/2 + 1/4
	1/4 + 1/2	1/4 + 1/2
Pseudomonas aeruginosa	1/2 + 1/4	1/2 + 1/4
	1/8 + 1/2	1/8 + 1/2
Candida albicans	1/2 + 1/16	1/2 + 1/16
	1/4 + 1/4	1/4 + 1/2

[a] Results obtained by the tube dilution method.

Results are given in Table IV, which shows the fractional portions of the minimum microbicidal concentrations of dyclonine hydrochloride and chlorobutanol which in combination produced a lethal effect. By the method employed, the action was considered additive if produced by a mixture of the drugs containing one-half the minimum lethal concentration of each agent when used alone; with greater than a 50% reduction in end point of one or both members, the effect was denoted as synergism (11–13). Against *Staphylococcus aureus*, for example, an additive action was shown by dyclonine hydrochloride and chlorobutanol at the end of twenty-four hours incubation, since one-half the minimum lethal concentration of each agent was required in combination for complete killing at this time. However, at forty-eight hours the drugs proved synergistic, with only one-fourth the minimum bactericidal concentration of chlorobutanol necessary in combination with one-half that of dyclonine hydrochloride for lethal action. Similar evidence of synergism between the two drugs was observed against *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Candida albicans*.

The effect of mixtures of dyclonine hydrochloride and chlorobutanol on the rate of killing was tested against *Staphylococcus aureus* and *Escherichia coli*. Test tubes containing 19 cc. of broth dilutions of the compounds singly and in various combinations were inoculated with 1 cc. of a diluted eighteen-hour culture of test organism, resulting in an initial

TABLE V.—EFFECTS OF COMBINATIONS OF DYCLONINE HYDROCHLORIDE AND CHLOROBUTANOL ON THE MULTIPLICATION OF STAPHYLOCOCCUS AUREUS

	Number of Organisms in Thousands per cc									
		Dyclonine HCl, %			Chlorobutanol, %			Dyclonine HCl, % + Chlorobutanol, %		
Hr.	Control	0.025	0.012	0.006	0.4	0.2	0.1	0.012 + 0.2	0.006 + 0.1	0.003 + 0.05
0	56.5	56.5	56.5	56.5	56.5	56.5	56.5	56.5	56.5	56.5
1	58	2.5	30.5	44	0	45	36	0.7	31	44
2	150	0.4	26.3	63	0	37	74	0.3	25.4	53
4	4,700	0.09	9.7	590	0	37	301	0.04	19	252
6	68,000	0.01	6.7	7,400	0	21	4,000	0.01	19.2	2,940
24	1,285,000	0	33,600	420,000	0	1,200	740,000	0	7,000	505,000

TABLE VI.—EFFECTS OF COMBINATIONS OF DYCLONINE HYDROCHLORIDE AND CHLOROBUTANOL ON THE MULTIPLICATION OF ESCHERICHIA COLI

	Number of Organisms in Thousands per cc.									
		Dyclonine HCl, %			Chlorobutanol, %			Dyclonine HCl, % + Chlorobutanol, %		
Hr.	Control	0.012	0.006	0.003	0.2	0.1	0.05	0.012 + 0.2	0.006 + 0.1	0.003 + 0.05
0	645	645	645	645	645	645	645	645	645	645
1	415	519	470	520	395	460	400	0.04	430	434
2	985	540	608	810	350	470	420	0	485	430
4	15,600	1,456	4,750	9,200	339	300	5,000	0	475	2,210
6	103,000	11,800	40,000	56,000	370	830	30,000	0	580	11,200
24	305,000	201,000	101,000	158,000	350	11,800	146,000	0	167	115,000

TABLE VII.—SELF-STERILIZING ACTION OF 0.5 PER CENT ISOTONIC AQUEOUS SOLUTION OF DYCLONINE HYDROCHLORIDE

Organism	No. of Cells Inoculated/cc. of Solution	Time in Minutes at Which Subculture Indicated Sterilization
Staphylococcus aureus	50	0–10
	500	0–10
Escherichia coli	100	0–10
	1,000	0–10
	10,000	0–10
Proteus vulgaris	150	0–10
	1,500	0–10
	15,000	0–10
Pseudomonas aeruginosa	60	0–10
	600	0–10
	6,000	10–30
Bacillus subtilis	250	0–10
	2,500	30–60
	25,000	120–240
Candida albicans	100	10–30
	1,000	60–120
	10,000	60–120

inoculum in the drug broth of 56,500 cells per cc. for the staphylococcus and 645,000 cells per cc. for *Escherichia coli*. Brain heart infusion broth was used for the former organism and the synthetic medium of Sahyun, *et al.* (8), for the latter. Using the end points previously found for dyclonine hydrochloride and chlorobutanol as guides, series of tubes were set up calculated to contain once, one-half, and one-fourth the minimum bactericidal concentration, respectively; and combinations of the drugs in concentrations of one-half plus one-half, one-fourth plus one-fourth, and one-eighth plus one-eighth the minimum lethal amounts of each agent. Tubes containing plain broth were inoculated with the test organisms as growth controls. All tubes were incubated in a water bath at 37°. At various intervals, 1-cc. samples were withdrawn, appropriate dilutions of which were plated out in brain heart infusion agar. Colony counts were made after incubation of plates for forty-eight hours.

Observations on the growth of *Staphylococcus aureus* and *Escherichia coli* in the various combinations of dyclonine hydrochloride and chlorobutanol are summarized in Tables V and VI. In interpreting data obtained by multiplication-time studies, two substances have been regarded as behaving synergistically when both acting in combination, each in a certain concentration for a certain time and under certain conditions on a fixed number of microorganisms, decrease the number to a lower level than does either of the agents acting alone at the concentration in which it was present in the combination and under the same conditions (14, 15). Synergism has been further defined as the ability of two drugs in combination to produce a marked increase in bactericidal rate over that possible with either drug alone, the outstanding characteristic being an immediate increase in killing rate by a mixture containing an apparently ineffective concentration of one agent and a somewhat active amount of the other (16, 17). From the results presented in Table V, it will be seen that combinations of dyclonine hydrochloride and chlorobutanol were more highly effective in inhibiting multiplication of the staphylococcus than were the same concentrations, respectively, of either agent alone. Further manifestation of synergism between the two substances was apparent with *Escherichia coli* (Table VI), rapid death of which occurred with a practically ineffective concentration of dyclonine hydrochloride (0.012%) in combination with an inhibitory amount of chlorobutanol (0.2%). Similar instances of increased bactericidal rates have been reported for various antibiotic mixtures (16, 18). Synergistic action of combinations of sulfonamides with antibiotics has also been reported (19–21).

Self-Sterilizing Properties.—The germicidal action of a 0.5% isotonic aqueous solution of dyclonine hydrochloride was determined after artificial contamination with the following microorganisms: *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Candida albicans*. Actively growing cultures were respectively inoculated in varying numbers, as indicated in Table VII, into 15-cc. volumes of the solution and held in a water bath at 37°. Subcultures were made into appropriate media at various intervals of time, incubated at 37°, and observed for growth after six days.

As will be seen in Table VII, the majority of aliquots of the dyclonine hydrochloride solution were sterile within ten minutes. In view of the increasing recognition of *Proteus* and *Pseudomonas* organisms as objectionable contaminants, especially in ophthalmic preparations, the results obtained with members of these two groups are noteworthy.

TABLE VIII.—BACTERICIDAL ACTION OF SOLUTION AND CREAM CONTAINING DYCLONINE HYDROCHLORIDE AND CHLOROBUTANOL AS DETERMINED BY THE WET FILTER-PAPER METHOD

Preparation	Growth of *Staphylococcus aureus* after Following Exposure Times, Minutes[a]						
	5	10	15	30	60	90	120
Aqueous Solution of Dyclonine HCl (1.4%) and Chlorobutanol (0.33%)	0	0	0	0	0	0	0
Aqueous Solution of Dyclonine HCl (1.4%)	+	+	+	+	+	+	+
Aqueous Solution of Chlorobutanol (0.33%)	+	+	+	+	+	+	+
Distilled Water	+	+	+	+	+	+	+
Cream Base with Dyclonine HCl (1.4%) and Chlorobutanol (0.33%)	+	0	0	0	0	0	0
Cream Base with Dyclonine HCl (1.4%)	+	+	+	+	+	0	0
Cream Base with Chlorobutanol (0.33%)	+	+	+	+	+	0	0
Cream Base	+	+	+	+	+	+	0

[a] Results after 96 hours incubation. All tubes showing no growth were negative for test organism on subculture.

Antiseptic Properties of Pharmaceutical Preparations Containing Dyclonine Hydrochloride.—Using the wet filter-paper method for testing antiseptics (10), studies were performed on an aqueous solution (*p*H 4.0)[2] and a nonionic vanishing cream,[2] each of which contained dyclonine hydrochloride at a concentration of 1.4% and chlorobutanol at 0.33%. The activity of each agent when present alone in the respective vehicles was similarly determined. Distilled water at *p*H 4.0 and the cream base were included as controls.

The test procedure was as follows: sterilized pieces (0.5 cm.²) of No. 2 Whatman filter paper were immersed in a twenty-four-hour broth culture of *Staphylococcus aureus*. A sufficient number of the inoculated paper squares were then placed into each of the preparations so that they were completely covered. At the end of five, ten, fifteen, thirty, sixty, ninety, and one hundred and twenty minutes' exposure at room temperature (28°), the respective papers were transferred to tubes containing 10 cc. of sterile broth. Following removal of adhering substance, the paper squares were retransferred to a second tube of sterile broth (10 cc.). All tubes were incubated at 37°. At the end of twenty-four hours, tubes showing no growth were subcultured into fresh sterile broth. Final readings on the original transfer tubes as well as on subcultures were made after ninety-six hours incubation.

Results are presented in Table VIII. By the wet filter-paper technique, it was observed that neither dyclonine hydrochloride nor chlorobutanol alone in aqueous solution was bactericidal after one hundred and twenty minutes' exposure of inoculated papers. The apparent decrease in bactericidal rate occurring with this procedure as compared to results obtained with the F. D. A. tube-method of antiseptic evaluation has been observed with other compounds (22, 23). It is believed that the severity of the filter-paper test accounts for the differences in results obtained with the two methods. In the tube procedure for phenol coefficient determination, as well as in the serial twofold dilution tests described above, the organisms are distributed throughout the solution, while in the filter-paper test the cells are confined to the paper square and hence exposed to only that portion of the solution with which it is in intimate contact. The longer time obviously required for antibacterial action with this test may thus be due to a larger inoculum involved, since, as indicated in the self-sterilizing experiments (Table VII), increases in the numbers of cells of several of the organisms exposed were followed by increases in the time necessary for sterilization. However, as shown in Table VIII, when dyclonine hydrochloride and chlorobutanol were combined, complete killing of *Staphylococcus aureus* occurred within five minutes. Data obtained with the cream preparations indicated that the base *per se* was bactericidal after one hundred and twenty minutes, with the addition of either dyclonine hydrochloride or chlorobutanol resulting in a killing time of ninety minutes in each case. Again, as with the solution, a marked increase in bactericidal rate was effected when the two agents were combined.

[2] Solution Dyclone; Creme Dyclone—Trade marks of Pitman-Moore Company, Division of Allied Laboratories, Inc., Indianapolis, Ind.

DISCUSSION

Data obtained in these studies indicate that dyclonine hydrochloride possesses significant antimicrobial activity. When used in a concentration of 0.5% or more in various pharmaceutical preparations, the drug, in addition to its anesthetic action, may be effective as a local antiseptic.

The preservation of pharmaceuticals has been a problem of long standing. Of importance in this regard was the self-sterilizing action shown by aqueous solutions of dyclonine hydrochloride following artificial inoculation with pathogenic microorganisms commonly involved in drug contamination. Sterility tests run on the cream formulation also indicated a freedom from bacteria capable of growing on blood agar and in brain heart infusion and thioglycollate broths. Some mold and yeast growth, representative of common airborne contaminants, has resulted, however, on Sabouraud agar. To a degree then, dyclonine hydrochloride should act as its own preservative. That this action may be augmented by chlorobutanol, considered in itself to be a satisfactory preservative (24, 25), is indicated by data obtained in the combination studies.

The presumption that dyclonine hydrochloride and chlorobutanol may act synergistically, based on the increase in phenol coefficient when the agents were combined, was confirmed by results obtained with the tube dilution tests and interval plating procedure. The greater action of the mixture in aqueous solution and in the cream, as shown by the more severe wet filter-paper method, added further evidence of a true synergistic relationship between the two drugs.

SUMMARY

1. Dyclonine hydrochloride, a topical anesthetic, has been found to possess significant antimicrobial activity.

2. Pharmaceutical preparations containing the drug have shown self-sterilizing properties which appeared to be increased in the presence of chlorobutanol.

3. A definite synergistic action was demonstrated with combinations of dyclonine hydrochloride and chlorobutanol against microorganisms commonly encountered in local infections.

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A Phytochemical Investigation of *Dryopteris Hexagonoptera* Michaux*

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The above ground portion of *Dryopteris hexagonoptera* Michaux and a dried aqueous extract of this plant, which have been reported to have hypotensive properties, were investigated. A glycosidal material was obtained from an alcohol extract of the plant; also from an aqueous extract. No filicin or alkaloids were found. A white crystalline material was isolated from a petroleum benzin extract of the plant, but not identified.

DRYOPTERIS SPECIES were known to Pliny and Galen as anthelmintics (1). Although *D. filix-mas* L. has been the most important single species, a number of others have been investigated for their properties and active principles (2–8). Most of the species examined contain anthelmintic phloroglucide compounds in varying amounts and in addition two investigators have reported the presence of a flavanol glycoside from *D. obligophlebia*.

In a private communication, J. Hudson Robinson, a physician of Charleston, West Virginia, reported that *D. hexagonoptera* has a hypotensive effect when the powdered above ground portion is administered in capsules or administered as an infusion. Since no previous work has been reported on this species, a study was made to compare the constituents in this plant with other *Dryopteris* species.

EXPERIMENTAL

Extraction of Whole Drug.—Ninety grams of ground drug was extracted successively in a Soxhlet apparatus with petroleum benzin (b. p. 30–60°), solvent ether, chloroform, and alcohol. Table I summarizes the results of this extraction.

TABLE I

Solvent	Wt. of Extract (Gm.)	% of Extract Obtained	Physical Appearance
Petroleum Benzin	3.8	4.3	dark green oily
Solvent Ether	2.7	3.0	brownish green
Chloroform	1.7	1.9	dark green aromatic
Alcohol	10.2	11.3	orange-brown aromatic

Petroleum Benzin Extract of Whole Drug.—The alcohol-soluble portion (1.45 Gm.–1.6% yield based on crude drug) of this fraction of the drug was refluxed with alcoholic potassium hydroxide U. S. P. for 10 minutes and then the unsaponifiable material was extracted with ether. Tests on this unsaponifiable extract for sterols by the digitonin precipitation method (9) were negative. Acidification of the reflux mixture changed the color of the solution from brown to green without precipitation. Ether extraction of this acidified solution yielded a negligible residue.

The alcohol-insoluble portion of the original extract when dissolved in petroleum benzin and treated with acetone yielded slim white rosette crystals (m. p. 64.0–64.5°) that were weakly polarizing with positive elongation but were not identified.

Ether Extract of Whole Drug.—In an attempt to determine the presence of phloroglucides the ether extract was triturated with 5 Gm. of MgO and 50 ml. of distilled water and then filtered. The filtrate was acidified to *p*H 3.5 ± 0.5 with hydrochloric acid. No precipitation was noted indicating the absence of

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phloroglucides. To check these findings, 100 Gm. of the aqueous extract, previously prepared from an infusion of the plant by drying in partial vacuum, was exhausted with ether by cold percolation. The residue obtained (4.1 Gm.) was extracted with an aqueous sodium carbonate solution (*p*H 9.0 ± 0.1), the resulting solution acidified and then extracted with ether. The residue after evaporation was refluxed with KOH in isobutanol and after extraction of the unsaponifiable material was acidified. The acidic solution was saturated with NaCl which caused separation of a small amount of sticky material soluble in, but not crystallizable from, alcohol, ethyl acetate, acetone, and 1% sodium carbonate solution, but insoluble in petroleum benzin and ether. A neutral aqueous solution gave a green color with 1% ferric chloride solution, a result reported to be obtained also with the phloroglucide, aspidinol.

Chloroform Extract of Whole Drug.—This extract was examined for alkaloids by the proximate assay method for alkaloids of the U. S. P. XIV (10) with the exception that 10% NaOH was used as the alkalinizing agent instead of ammonia. An acid solution of the resulting extract gave positive tests with gold chloride solution (1%) and Mayer's reagent but no reaction with Lugol's solution or phosphomolybdic acid solution (1%). Because of these inconclusive tests an attempt to isolate alkaloids was carried out on a larger sample of the crude drug.

Ammoniacal chloroform was used to exhaust 5.145 Kg. of a No. 30 powder of the crude drug in a Lloyd extractor. The chloroform extract was treated as outlined above but no alkaloids could be isolated.

Alcohol Extract of Whole Drug.—The alcohol extract was dissolved in 100 ml. of alcohol and enough water added to reduce the alcohol strength to 40% v/v. The green precipitate was removed by filtration and discarded. The deep amber filtrate was precipitated with excess lead acetate solution [20% $Pb(OOCCH_3)_2 \cdot 2H_2O$] and filtered, the precipitate being discarded. The filtrate obtained was then treated with an excess of saturated basic lead acetate solution and filtered. The precipitate obtained was saved and will be discussed later.

The filtrate was freed of lead ions by treatment with excess saturated sodium biphosphate solution. This lead-free solution was saturated with sodium chloride and exhausted with ether. The resulting alcohol-ether-water solution was shaken with distilled water and the aqueous-alcohol solution then saturated with sodium chloride. This salt-saturated solution was exhausted with ether and the ethereal extract evaporated. The brown syrupy residue which was possibly glycosidal material was too small an amount to work with, so an attempt was made to obtain a larger quantity from the dried aqueous extract.

Twenty grams of an aqueous extract, made from an infusion of the whole drug and subsequent drying, was treated with neutral lead acetate as described above. After filtering out the precipitate and removing the excess lead with H_2S the lead-free solution was azeotropically distilled with isobutanol at about 40° to remove the water. The isobutanol-insoluble residue resulting was treated with methanol which dissolved a brown, sticky, very bitter material with no definite melting point, soluble in acetone, ethyl acetate, and water, and insoluble in petroleum benzin, ethanol, chloroform, and ether. Attempts to purify this material by crystallization from its solvents were unsuccessful.

The methanol-insoluble portion after azeotropic distillation appeared as brown contaminated crystals soluble in ethyl acetate, acetone, and water, but insoluble in petroleum benzin, chloroform, ether, and ethanol. The brown contaminant could not be removed by using any of the solvents. An aqueous solution of the impure crystals gave a positive Benedict's test before and after heating with an equal volume of 10% HCl.

The residue obtained in the basic lead acetate precipitation outlined above was mixed with enough water to make a thin slurry and the lead removed by treatment with H_2S. The filtrate obtained after filtering off the lead sulfide was evaporated and produced a small amount of a brownish oily material which was soluble in alcohol, acetone, and ethyl acetate, but not crystallizable from any of these.

SUMMARY

A phytochemical investigation of *Dryopteris hexagonoptera* Michaux, a plant reported to have hypotensive properties, was carried out to compare the principles of this plant with other *Dryopteris* species. Following are the results.

1. No phloroglucide products similar to those reported in other *Dryopteris* species could be found, except a qualitative test for aspidinol. The absence of filicin is important if the drug is to be used as a hypotensive agent.

2. Although some positive tests with alkaloidal reagents were obtained no alkaloids could be isolated.

3. An impure crystalline material giving a presumptive test for glycosides was isolated. Positive identification of a glycoside is lacking but strongly indicated by the unusual solubility of this material.

4. No sterols capable of precipitation with digitonin were found.

5. From a petroleum benzin extract a white material (m. p. 64.0–64.5°) that formed slim rosette-shaped crystals weakly polarizing with positive elongation was obtained.

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Fungistatic, Bacteriostatic, and Amebicidal Studies on the Isomeric Normal Nonynoic Acids*

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The position of unsaturation in the isomeric normal nonynoic acids was shown to have some bearing on fungistatic activity. There is reasonable agreement with the fungistatic activity of the isomeric normal octynoic acids. In general, the nonynoic acids have higher fungistatic properties than the octynoic acids, but neither are comparable to the overall activity of undecylenic acid. The general bacteriostatic effect *in vitro* of the nonynoic acid isomers was shown to be negligible and contrary to the effect of the octynoic acid isomers. However, both 2- and 4-nonynoic acids were shown to have some effect in inhibiting the growth of *Mycobacterium tuberculosum*. The *in vitro* amebicidal activity of the isomeric nonynoic acids was found to be of a rather low grade. The *in vivo* use of 2-nonynoic acid was shown to allow a higher degree of infection in the test animal than in the control animals.

THE POSITION of the triple bond with respect to the carboxyl group was shown to affect the fungistatic and bacteriostatic properties in the isomeric normal octynoic acids (1). The recent synthesis of the isomeric normal nonynoic acids (2, 3) furnished another series of acetylenic acids for comparative testing of the related even (eight) and odd (nine) carbon acids.

EXPERIMENTAL

Fungistatic Activity.—Brain heart infusion agar (Difco) (4) was the medium used in all tests. Since heating may alter the *p*H of the medium, it was prepared and sterilized prior to adding the test acids, adjustment of *p*H and making of dilutions, which were carried out aseptically. Samples of the acids were weighed out and placed in 500-cc. graduated cylinders, moistened with sodium hydroxide to make soluble, and dissolved in 360 cc. molten agar medium at 45°. After thorough mixing this material was divided into four equal parts in 250-cc. flasks and held in the 45° water bath to prevent solidification. These portions were adjusted respectively to *p*H 5, 6, 7, and 8 with normal sodium hydroxide or hydrochloric acid and brought up to volume to make a concentration of 100 mg. test acid per 100 cc. Dilutions of each acid were prepared with molten agar, without test acids and adjusted to the corresponding *p*H levels, to give concentrations of 50, 20, 5, and 1 mg. per 100 cc. of medium. Each dilution was then dispensed into 13 × 100 mm. tubes, using 2 cc. per tube, slanted and allowed to harden.

The slants were inoculated by streaking a loopful of inoculum (saline suspensions of fungus spores) across the surface of the slant. The tubes were incubated at 28° and were read for growth (no inhibition) or no growth (inhibition) after 3–8 days depending on the species of organism. The test species and the days incubated were: *Nocardia asteroides*, 7; *Allescheria boydii*, 3; *Aspergillus fumigatus*, 3; *Blastomyces dermatitidis*, 3; *Candida albicans*, 3; *Cryptococcus neoformans*, 3; *Histoplasma capsulatum*, 7; *Hormodendrum pedrosoi*, 7; *Microsporum audouini*, 3; *Microsporum gypseum*, 3; *Sporotrichum schenckii*, 3; *Trichophyton mentagrophytes*, 3; *Trichophyton rubrum*, 3.

All determinations were in triplicate, except as noted, and the results (which were in good agreement in every case) were averaged to obtain the data shown in Table I. The 8-isomer was tested against six organisms only.

Overall Comparative Activity.—Because of its known antifungal properties, undecylenic acid was used as the standard in these comparative studies. As shown in Table I, low concentrations of undecylenic acid inhibit the growth of all fungi tested, especially in the acid *p*H range. The 4-, followed by the 8-isomer of nonynoic acid showed the greatest antifungal activity. The 2-isomer exhibited the least activity, while the other isomers (5-, 6-, and 7-) had an intermediate activity.

At *p*H 6, the optimum *p*H for the growth of most of the test fungi, isomer 4- was superior to the other isomers against organisms 2, 7, 11, and 14; superior to all but one of the others against organisms 4 and 6; equal to all others against organism 5; and less active than at least one of the other isomers against organisms 1, 3, 8, 10, 12, and 13. Isomer 4- was more active against *Histoplasma capsulatum* (7) and less active for the others than undecylenic acid. Growth of organism 9 was poor at *p*H 6, and comparison could not be made.

Comparative Activity with Octynoic Acids.—Comparative studies with the octynoic acids were completed only on five fungi at the *p*H of 6 and 7 (1). The overall picture of activities is shown in Table II. At the *p*H of 7, the activity of the nonynoic acids matches or excels (in most cases) the activity of the octynoic acids. At the *p*H of 6, the octynoic acids showed slightly higher activity against *Histoplasma capsulatum* (organism 7). At this *p*H, the activity

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‡ Research Laboratories, Parke, Davis and Company, Detroit, Michigan. The author wishes to acknowledge the supervision of Dr. Arthur B. Hillegas during the fungistatic testing.

§ Contribution No. 953 from the Department of Chemistry, University of Pittsburgh. The author wishes to acknowledge the cooperation of Dr. Alexander M. Moore, Parke, Davis and Company, Multiple Fellowship in Medicinal Chemistry at Mellon Institute.

TABLE I.—CONCENTRATIONS (MG./100 ML.) OF UNDECYLENIC AND NONYNOIC ACIDS REQUIRED FOR INHI

Organism	Inhibition[a] pH	Undecylenic Acid 5	6	7	8	2-Nonynoic Acid 5	6	7	8	4-Nonynoic Acid 5	6	7	8
1. *Nocardia asteroides*	C	NGC[c]	1	10	50	NGC	5	50	50[d]	NGC	5	50	50[d]
	P			5			1	20	50[d]		1	10	50[d]
2. *Allescheria boydii*	C	1	5	5	20	20	50[d]	50[d]	50[d]	5	20	50[d]	50[d]
	P		1		10	10	50	50[d]	50[d]	1	10	50	50[d]
3. *Aspergillus fumigatus*	C	10	10	50	50[d]	50[d]	50[d]	50[d]	50[d]	20	50[d]	50[d]	50[d]
	P	5		20	50	50	50[d]	50[d]	50[d]	10	50	50[d]	50[d]
4. *Blastomyces dermatitidis*[b]	C	5	5	10	20	20	50	50[d]	50[d]	10	10	50	50[d]
	P	1	1	5	5	5	20	50[d]	50[d]	1	5	20	50[d]
5. *Candida albicans*	C	10	50	50[d]	50[d]	50[d]	50[d]	50[d]	50[d]	20	50[d]	50[d]	50[d]
	P	5	20	50	50	50[d]	50[d]	50[d]	50[d]		50[d]	50[d]	50[d]
6. *Cryptococcus neoformans*	C	NGC	5	10	NGC	NGC	50[d]	50[d]	NGC	NGC	50	50[d]	NGC
	P			5			50[d]	50[d]				50[d]	
7. *Histoplasma capsulatum*	C	NGC	5	10	20	NGC	20	50[d]	50[d]	NGC	1	10	20
	P		1	5	5		5	50	50[d]			1	
8. *Hormodendrum pedrosoi*	C	5	20	50	50	50	50[d]	50[d]	50[d]	20	50	50[d]	50[d]
	P		5	5	10	20	20	50[d]	50[d]	10	10	10	50[d]
9. *Microsporum audouini*	C	1	1	1	10	1	20	50[d]	50[d]	NGC	1	20	1
	P				1		5	50	50[d]			1	
10. *Microsporum canis*[b]	C	1	1	5	5	20	50[d]	50	50	NGC	10	20	1
	P			1	1	1	50[d]				5	10	
11. *Microsporum gypseum*	C	1	5	10	50	50	50[d]	50[d]	50[d]	5	20	50[d]	50[d]
	P			5	20	10	50	50[d]	50[d]	1	5	50	50[d]
12. *Sporotrichum schenckii*	C	5	5	20	50	50[d]	50[d]	50[d]	50[d]	10	50[d]	50[d]	50[d]
	P	1		5	20	50	50[d]	50[d]	50[d]	5	50	50[d]	50[d]
13. *Trichophyton mentagrophytes*[b]	C	1	5	5	1	10	50[d]	50	10	1	20	50	50
	P		1	1		5	50				10	20	20
14. *Trichophyton rubrum*	C	1	5	10	50	10	50[d]	50[d]	50[d]	5	10	50	50[d]
	P		1	5	20	5	50	50[d]	50[d]	1	5	20	50[d]

[a] The symbols indicate complete (C) or partial (P) inhibition by the corresponding concentrations (mg./100 ml.) shown
pound on organism at this *p*H could not be estimated. [d] Greater than.

of 7-octynoic acid on organism 7 almost equalled that of the standard undecylenic acid. It is interesting to note that the highest activity of the nonynoic acids against this same organism is also exhibited by the 7-isomer. Against *Blastomyces dermatitidis* (organism 4) the nonynoic acids showed slightly higher activity than the octynoic acids. The highest unit activity at *p*H 6 was exhibited by 7-nonynoic acid against *Microsporum canis* (organism 10). The best overall activity at *p*H 6, however, was shown by 4-nonynoic acid, which, in all cases where control growth occurred, showed inhibition against each organism. It is interesting to note, also, that at *p*H 7, 8-nonynoic acid showed more activity against organism 13 than the standard, undecylenic acid. In fact, the overall picture of activity by this 8-isomer was good, and may seem to be better than the 4-isomer. The lack of all the data concerning the 8-isomer makes it questionable as to whether it really is more active than the 4-isomer.

In conclusion, it seems that the 4-isomers of both acids showed high overall activity, followed by the terminal isomers (7-octynoic; 8-nonynoic). The isomers with unsaturation next to the terminal carbon (6-octynoic; 7-nonynoic) also showed a general high activity. Undecylenic acid, however, was, by far, more active than either the octynoic or the nonynoic acids.

Activity of the Methyl Ester and Amide of 4-Nonynoic Acid.—The methyl ester and amide of 4-nonynoic acid were tested at *p*H 7 against the three organisms, as shown in Table III. The amide and ester are identical in activity, but less effective than the free acid.

Bacteriostatic Activity.—*General.*—The 2, 4, 5, 6, 7, and 8 isomers of nonynoic acid were evaluated against *Escherichia coli, Neisseria catarrhalis, Proteus vulgaris, Pseudomonas aeruginosa, Shigella paradysenteriae var. Flexner Z., Streptococcus hemolyticus, Brucella suis,* and *Salmonella typhosa* by the agar-plate-disk method.[1] None of the compounds showed an appreciable effect on the growth of any of the organisms used even at concentrations as high as 100 mg. per 100 ml.

Comparative results for the octynoic acids (1) are available for *Salmonella typhi* and *Shigella paradysenteriae var. Flexner Z.* All the octynoic acid isomers showed some antibacterial activity on the bacteria tested.

Tuberculosis.—The evaluation *in vitro*[2] of the 2- and 4-nonynoic acids against the virulent H37RU strain of *Mycobacterium tuberculosis var. hominis* showed that both compounds had some effect in inhibiting the growth of the organism. The least amount of acid (mg./100 cc.) which completely inhibits growth in simple medium was 2.5 for 2-nonynoic acid and 1.25 for 4-nonynoic acid. At a concentration of 10 mg./100 cc. in simple medium and ten per cent bovine serum, there was growth of the organism for the 2-nonynoic acid, and no growth for the 4-nonynoic acid. It is interesting to note that the higher antifungal activity of the 4-isomer is supported by the antitubercular data.

[1] The authors wish to acknowledge this antibacterial screening by Dr. A. S. Schlingman, Research Department, Parke, Davis and Co., Detroit, 32, Michigan.

[2] The authors wish to acknowledge this tubercle bacillus screening by Guy P. Youmans, Northwestern University Medical School.

BITION OF PATHOGENIC FUNGI *in Vitro*

5-Nonynoic Acid				6-Nonynoic Acid				7-Nonynoic Acid				8-Nonynoic Acid			
5	6	7	8	5	6	7	8	5	6	7	8	5	6	7	8
NGC	1	50[d]	50[d]	NGC	5	50	50[d]	NGC	20	50[d]	50[d]				
		50	50[d]		1	20	50[d]		1	50					
10	50	50[d]	50[d]	10	50	50[d]	50[d]	10	50[d]	50[d]	50[d]				
	20	50[d]	50[d]		10	50[d]	50[d]	5	50						
50	50	50[d]	50[d]	50	50	50[d]	50[d]	50	50[d]	50[d]	50				
20		50	50[d]			50[d]	50[d]	20	50						
5	10	50[d]	50[d]	5	20	50	50[d]	5	20	50[d]	50[d]	5	20	50	50[d]
		50[d]	50[d]		5	20	20		10	50	50[d]	1	10	20	50[d]
50	50[d]	50[d]	50[d]	50	50[d]	50[d]	50[d]	50	50[d]	50[d]	50[d]	50	50[d]	50[d]	50[d]
	50	50[d]	50[d]		50	50[d]	50[d]	20				20			
NGC	50	50[d]	NGC	NGC	50[d]	50[d]	NGC	NGC	50[d]	50[d]	NGC				
		50[d]			50	50[d]			50	50					
NGC	10	50	50[d]	NGC	5	50	50[d]	NGC	20	50[d]	50[d]	NGC	10	5	50
	1		50			10	20			50	50		5	1	20
10	50	50[d]	50[d]	20	20	50[d]	50[d]			50[d]	50[d]				
5	20	50	50[d]	10		20	50[d]	20	50						
NGC	NGC	50	50[d]	NGC	NGC	50	NGC	NGC	NGC	50	NGC				
		20	50			20				20					
NGC	10	50[d]	20	NGC	5	20	20	NGC	5	50	50[d]	NGC	5	20	50[d]
	1	10	10		1	5	10		1	20	50		1	10	50[d]
10	20	50[d]	50[d]	5	20	50[d]	50[d]	5	50	50[d]	50[d]				
5	10	50[d]	50[d]			50	50[d]	1	10	50					
20	50	50[d]	50[d]	20	50	50[d]	50[d]	20	50[d]	50[d]	50[d]	10	50	50[d]	50[d]
5	20	50[d]	50[d]	10		50[d]	50[d]	10	20	50	50	5	20	50	20
5	50	20	10	5	20	20	10	5	50	20	10	1	10	1	10
		10	5	1	10		5	1	10	10	5		5		5
5	20	50[d]	50[d]	10	50	50[d]	50[d]	5	20	50[d]	50[d]				
	10	50	50[d]		20	50[d]	50[d]		1	20	50[d]				

in the table. [b] Results based on duplicate tubes. [c] NGC—no growth in control tube. Accordingly, effect of com-

TABLE II.—CONCENTRATIONS (MG./100 ML.) OF OCTYNOIC, UNDECYLENIC AND NONYNOIC ACIDS REQUIRED FOR INHIBITION OF FUNGI *in Vitro*

			Octynoic Acids[a] Isomer						Undecylenic	Nonynoic Acids Isomer						
	Organism	pH	2	3	4	5	6	7	Acid	2	3	4	5	6	7	8
7	*Histoplasma capsulatum*	6	50[b]	10	10	20	10	1[b]	1	50	20	10	20	20	5	10
		7	50[b]	50	50	50[b]	20	10	5	50[b]		20	50[b]	50	50	5
10.	*Microsporum canis*	6	50[b]	50[b]	50	50	50	50[b]	1	50[b]		10	10	1P	1	1P
		7	50[b]	50[b]	50[b]	50[b]	50[b]	50[b]	5	50		20	50	20	50	20
12.	*Sporotrichum schenckii*	6	50[b]	50[b]	50	50	50[b]	50[b]	5	50*P*		50P	50P	50P	50P	50
		7	50[b]	50[b]	50[b]	50[b]	50[b]	50[b]	20	50[b]		50[b]	50[b]	50[b]	50[b]	50P
4.	*Blastomyces dermatitidis*	6	50[b]	20	10	20	10	50	5	50	20	10	10	20	20	20
		7	50[b]	50[b]	50	50[b]	50	50[b]	10	50[b]		50	50[b]	50	50P	50
13.	*Trichophyton mentagrophytes*	6	50[b]	50[b]	50	50	50	50[b]	5	50P	40[b]	20	50	20	50	10
		7	50[b]	50[b]	50[b]	50[b]	50[b]	50[b]	5	50		50	20	20	20	1

[a] Fungistatic data on octynoic acids reference (1) except data on 3-nonynoic acid, which is from unpublished data of Cox (6). [b] Greater than.

TABLE III.—CONCENTRATIONS (MG./100 ML.) OF THE METHYL ESTER AND AMIDE OF 4-NONYNOIC ACID REQUIRED FOR INHIBITION OF FUNGI *in Vitro*

		Methyl Ester Inhibition			Amide Inhibition		
Fungus	No.	Complete	Partial	None	Complete	Partial	None
Candida albicans	5	..	..	50	..	..	50
Tricophyton mentagrophytes	13	50	20	10	50	20	10
Histoplasma capsulatum	7	..	..	50	..	..	50

Amebicidal Activity.[3]—*In Vitro.*—The evaluation of the 2, 4, 5, 6, 7, and 8-nonynoic acids against the pathogenic *Endamoeba histolytica in vitro* showed that all the compounds, with the exception of the 7-isomer, showed activity, although of a rather low

[3] The authors wish to acknowledge all the amebicidal screening by Dr. Paul E. Thompson, Research Department, Parke, Davis and Company.

TABLE IV—EFFECT OF ISOMERS OF NONYNOIC ACID ON ENDAMOEBA HISTOLYTICA *In Vitro*

Isomer	Effect of Drug at the Following Dilutions		
	1:500	1:1000	1:5000
2	..	Cidal	Inactive[a]
4	Cidal	Static	Inactive
5	Cidal	Inactive[a]	..
6	..	Cidal	Inactive
7	Inactive[a]	..	..
8	Cidal	Inactive[a]	..

[a] Suppression, but not static.

grade. The data are tabulated in Table IV. The testing procedure was described previously (5).

In Vivo.—The evaluation of 2-nonynoic acid against intestinal amebiasis in the rat showed that the average degree of infection in the animals treated with 36 mg./Kg. daily for seven days was actually higher than in the control animals. It was suggested that higher levels of drug be used, but it had been found that 470 mg./Kg. daily in mice was close to the maximum tolerated dose.

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(6) Cox, C. D., unpublished data.

Comparison of Aqueous and Repository Tubocurarine for Muscle Relaxant Effectiveness and Toxicity in Rabbits*

By HAROLD BLUMBERG, ANNETTE L. KALLENBACH, HYMAN B. DAYTON, and SAMUEL M. GORDON

A comparison was made of aqueous tubocurarine and a repository *d*-tubocurarine chloride suspension (Tubadil®) for muscle relaxant effectiveness and toxicity in rabbits. As compared with intravenous aqueous tubocurarine on the basis of lethal dose, intramuscular aqueous tubocurarine was one-half as toxic, intravenous Tubadil one-third as toxic, and intramuscular Tubadil only one-tenth as toxic. Deaths from aqueous tubocurarine occurred very soon after injection, whereas those from Tubadil were delayed, occurring after a prolonged period of gradually deepening respiratory depression. Duration of muscle relaxation was determined at sublethal dosages which produced the same average maximum severity of reaction, i. e., complete ataxia, and therefore had the same degree of safety. The muscle relaxation achieved with intramuscular Tubadil was 8 times as long as with intramuscular aqueous tubocurarine and 30 times as long as with intravenous aqueous tubocurarine. Overcurarization with Tubadil was counteracted by Tensilon® or neostigmine, the longer-acting neostigmine appearing more effective.

AQUEOUS SOLUTIONS of tubocurarine have been used intravenously for more than a decade to produce short, rapid relaxation of skeletal muscle, particularly in abdominal surgery. The hazard of respiratory failure inherent in this procedure necessitates constant vigilance to guard against untoward incidents. For many other therapeutic purposes, however, a gradual, sustained relaxation of skeletal muscle is desirable.

The wide use of curare as an adjunct to general anesthetics caused many to overlook the report of Burman (2), who in 1938 used purified extracts of curare for the relief of skeletal muscle spasm in organic neurological disorders. A few years later Bennett (3) commented that the action of aqueous solutions of curare was too fleeting for this purpose. Consequently he stated that a slow-acting, sustained effect was necessary for the drug to become a useful therapeutic aid in these conditions. The achievement of this aim was made possible by the pioneering work of Gill (4) in the commercial scale preparation of pure tubocurarine chloride. From this resulted the development of intramuscular repository suspensions of tubocurarine capable of producing prolonged muscular relaxation and requiring less observation of the patient than is needed with

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Presented in part to the Scientific Section, A. PH. A., Boston meeting, August, 1954, and in part to the American Society for Pharmacology and Experimental Therapeutics, San Francisco meeting, April, 1955 (1).

the aqueous tubocurarine. High chemical purity is a primary consideration for the preparation of a repository injection, since an accurate chemical method can be applied for standardization (5). The biological methods of assay are not readily applicable to repository suspensions.

A slow-acting repository preparation, Tubadil®, has been reported to be useful in the treatment of many clinical conditions involving skeletal muscle spasm and pain, such as traumatic injuries (6–10), arthritides other than rheumatoid (8), in physical rehabilitative maneuvers (11, 12), tetanus (13, 14), and as a pre- and postoperative relaxant for anorectal and other types of surgery (15–18).

In connection with the first clinical investigation of Tubadil, Fuller (6) cited the experiments of Neff showing the comparative safety of Tubadil injected intramuscularly into dogs. The present report compares aqueous tubocurarine and Tubadil for muscle relaxant effectiveness and toxicity or safety in rabbits. Rabbits were chosen, since they are used in the biological assay of tubocurarine chloride injection, U. S. P.

EXPERIMENTAL

Materials and Methods.—The repository tubocurarine preparation has the following composition per cc.: *d*-tubocurarine chloride pentahydrate 25 mg., oxycholesterol derivatives 312 mg., peanut oil 562 mg., white wax 20 mg., and chlorobutanol 5 mg. For purposes of brevity this will be referred to as Tubadil. The aqueous solutions contained 3 mg./cc., expressed as *d*-tubocurarine chloride pentahydrate, and were made from the same batch of tubocurarine that was used for preparing the repository form. Edrophonium chloride (Tensilon® chloride) and neostigmine methylsulfate (Prostigmin® methylsulfate) were commercial vials with concentrations of 10 mg./cc. and 1 mg./cc., respectively.

Young adult female New Zealand white rabbits, weighing 2.5 to 3.5 Kg., were used. Intravenous injections of the aqueous solutions were made in the marginal ear vein; the intramuscular injections were made into the gastrocnemius muscle. With Tubadil the intravenous injection was given very slowly in the marginal ear vein, whereas the intramuscular injection was given in the gastrocnemius muscle with a 20-gauge needle. The Tubadil was liquefied by warming in the hand and shaking before withdrawal.

After injection the rabbits were allowed to remain unrestrained in large cages to permit full observation of the signs of curarization. The animals were disturbed as little as possible in order to minimize the effect of voluntary movement in augmenting the muscle relaxant response to tubocurarine. The severity of the curarization was scored as shown in Table I.

Toxicity.—The acute toxicity of tubocurarine, as measured by the LD_{50}, was compared in the following four variations of menstruum and injection route: intravenous aqueous tubocurarine, intramuscular aqueous tubocurarine, intravenous Tubadil, and intramuscular Tubadil. As shown by the LD_{50} values in Fig. 1, intramuscular Tubadil was only one-tenth as toxic as intravenous aqueous tubocurarine. The value found for the LD_{50} of intravenous aqueous tubocurarine, 0.20 mg./Kg., agrees very well with the figures of previous investigators, Marsh and Pelletier (19) and Hoppe (20). The LD_{50} for intramuscular aqueous tubocurarine was about twice that for the intravenous, which is consistent with Everett's findings (21) that the rabbit head drop dose by intramuscular injection was about twice the intravenous head drop dose. It was noteworthy that intramuscular Tubadil was only about one-fifth as toxic as intramuscular aqueous tubocurarine, thus demonstrating that the decreased toxicity of intramuscular Tubadil was not due to the method of injection but instead to the slow absorption from the menstruum.

Table I.—Sequence of Curarization Responses in Rabbits

Score	Reaction
1	Stamping of hind legs; hind leg weakness
2	Ataxia hind legs
3	Complete ataxia, fore and hind legs; cannot stand
4	Weak neck, but head rising to stimulus
5	Head drop, not rising to stimulus; moderate respiratory depression
6	Body flaccid
7	Respiration slow and irregular
8	Cyanosis
9	Death

Similarly, when Tubadil was administered intravenously by slow injection, it was still only about one-third as toxic as intravenous aqueous tubocurarine. This demonstrated again that the lowered toxicity of Tubadil was due to the slow absorption from the menstruum. At lethal doses the rabbits that received intravenous Tubadil showed the typical progression of signs of overcurarization, indicating that the deaths were due to overdosage of tubocurarine and not to fat embolism.

Duration of Signs of Curarization Before Death.—The time sequences of reactions to aqueous tubocurarine and to Tubadil were compared to determine the duration of signs of curarization before death, i. e., the rapidity of the occurrence of death after the detection of the first sign of overcurarization. For this purpose comparative calculations were made on rabbits which died at approximately the LD_{50} in each group. Ten rabbits were used for each of the 4 groups. The average time in minutes, and standard error, were computed for: time from injection to death (D); time from injection to first sign of curarization (S); and time from first sign of curarization to death (D minus S). Since the early subjective clinical signs of curarization, such as lassitude, cannot be used in experimental animals, the first objective sign of curarization was taken as leg weakness, indicated by stamping of the hind legs.

The data in Fig. 2 show that there is a slight or moderate lengthening of the induction period of curarization as the injection goes from intravenous aqueous tubocurarine to intramuscular aqueous

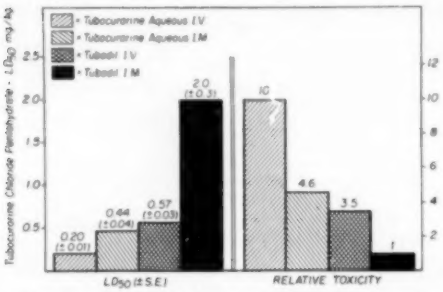

Fig. 1.—Acute toxicity of tubocurarine in aqueous and repository menstruum (Tubadil) in rabbits. 50 rabbits for each determination.

tubocurarine, to intravenous Tubadil, to intramuscular Tubadil. However, in the same sequence there is a very great increase in the time from the first sign of curarization to death. Thus, with intravenous aqueous tubocurarine death supervened in an average of 3.4 ± 0.2 minutes after the first sign of curarization, whereas after intramuscular Tubadil there was a lapse of 78.5 ± 19.8 minutes before death. The times for the other two modes of administration were intermediate, namely, intramuscular aqueous tubocurarine 7.5 ± 1.1 and intravenous Tubadil 18.9 ± 2.9 minutes.

Therefore, at lethal doses the progression of the overcurarization was much slower and gradual after intramuscular Tubadil than after intravenous aqueous tubocurarine. With intramuscular Tubadil the warning period from the appearance of objective signs of curarization to death was about 20 times as long as with intravenous aqueous tubocurarine. This meant that with intravenous aqueous tubocurarine any counteractant or antidotal injection had to be administered within about 3 minutes or it would be too late, while with intramuscular Tubadil there was a safety or lag period of about 78 minutes, during which counteractant injections or other measures could be used.

Duration of Muscle Relaxation at Nonlethal Levels.—The duration of the desired therapeutic effect, namely, relaxation of skeletal muscle, was determined by injection of rabbits at the maximum nonlethal levels of the preparations. These dosages were: intravenous aqueous tubocurarine 0.14, intramuscular aqueous tubocurarine 0.28, and intramuscular Tubadil 1.25 mg./Kg. Groups of 10 rabbits were used, with the same average maximum severity of reaction, i. e., between complete ataxia and weak neck (actual score of 3.3), and therefore with the same degree of safety.

The duration of objective muscle relaxation, i. e., from first leg weakness to recovery, was (mean ± S.E.): intravenous aqueous tubocurarine 8.2 ± 0.7, intramuscular aqueous tubocurarine 29 ± 2, and intramuscular Tubadil 246 ± 36 minutes (see Fig. 3). Therefore, with the same degree of safety, the muscle relaxation achieved with intramuscular Tubadil was 8 times as long as with intramuscular aqueous tubocurarine and 30 times as long as with intravenous aqueous tubocurarine.

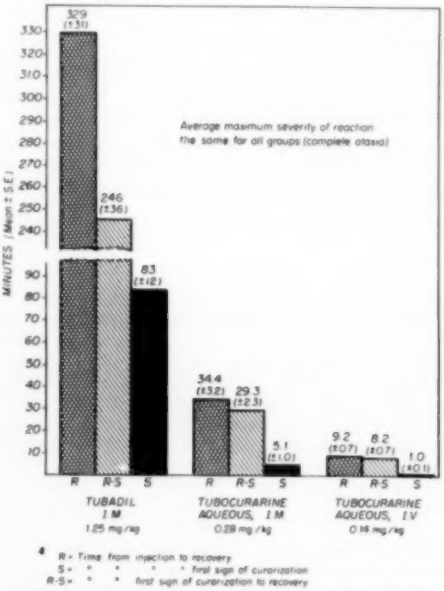
MINUTES (Mean ± S.E.)
Average maximum severity of reaction the same for all groups (complete ataxia)
329 (±31)
246 (±36)
83 (±12)
34.4 (±3.2)
29.3 (±2.3)
5.1 (±1.0)
9.2 (±0.7)
8.2 (±0.7)
1.0 (±0.1)
R R-S S
TUBADIL I.M. 1.25 mg./kg
TUBOCURARINE AQUEOUS, I.M. 0.28 mg./kg
TUBOCURARINE AQUEOUS, I.V. 0.14 mg./kg
R = Time from injection to recovery
S = " " " first sign of curarization
R-S = " " first sign of curarization to recovery

Fig. 3.—Duration and recovery from curarization in rabbits after aqueous and repository tubocurarine (Tubadil) at nonlethal levels.

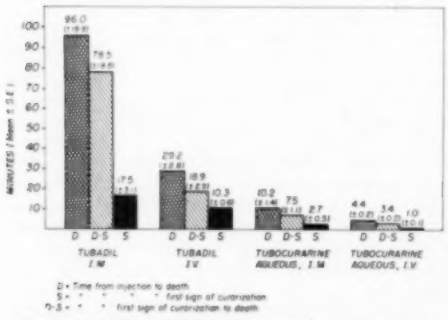

Fig. 2.—Time sequence of reactions to aqueous and repository tubocurarine (Tubadil) in rabbits (at approximately LD_{50}).

Counteraction of Tubadil by Tensilon and Prostigmin.—Curarization with Tubadil was counteracted by Tensilon chloride or Prostigmin methylsulfate. The objective curarization was produced by Tubadil at 1.25 mg./Kg. intramuscularly. At the first definite sign of leg weakness or hind-leg ataxia, the rabbits were injected intramuscularly or intravenously with Tensilon at 1 mg./Kg. or Prostigmin at 0.1 mg./Kg. These dosages represent the clinical ratios of the drugs in curare counteraction. Atropine sulfate was given at 1 mg./Kg. intramuscularly to guard against any undesirable muscarinic effects of the two antidotes. Groups of 20 rabbits were used, the animals being crossed over after a one-week interval so as to serve as their own controls.

The longer acting Prostigmin seemed more adequate against the prolonged action of Tubadil. The

ataxia was counteracted and normal leg movement maintained for an average of 47 minutes after Tensilon as compared with 84 minutes after Prostigmin. As expected, the tubocurarine counteraction was most rapid after intravenous Tensilon. When the animals subsequently relapsed into ataxia, they again responded to injections of Tensilon or Prostigmin by regaining normal leg movement. Here again Prostigmin showed a more lasting effect, since over an observation period of 3 hours the Tensilon animals required an average of 3 injections, while the Prostigmin animals required an average of 1.2 injections.

DISCUSSION

The results of these experiments on rabbits may be considered in relation to the dosages of aqueous tubocurarine and of Tubadil that are used clinically. The recommended single dose of intravenous aqueous tubocurarine chloride in surgery is 6–9 mg. (22), whereas a single intramuscular dose of Tubadil is 1–2.5 cc., equivalent to 25–62.5 mg. of tubocurarine chloride. Thus, about 4–7 times as much tubocurarine is ordinarily administered in the form of Tubadil, as compared with aqueous tubocurarine. This increased clinical dosage is in agreement with the rabbit experiments herein described, in which the toxicity of the gradually acting intramuscular Tubadil was only one-tenth that of intravenous aqueous tubocurarine, as determined by the LD_{50}.

It is interesting to relate the survival of the rabbits at the highest nonlethal dose of tubocurarine chloride in Tubadil, 1.25 mg./Kg. intramuscularly, to certain instances of overdosage in humans. Five cases of overdosage with Tubadil have been reported by Hedge (23). In each case 5 cc., or 125 mg. of tubocurarine chloride, was injected. No fatality occurred, even though the usual routine curare antidotes and resuscitative measures were not employed in all 5 cases. For a 70-Kg. individual this is a dose of 1.8 mg./Kg., as compared with the maximum nonlethal dose of 1.25 mg./Kg. found in the rabbit experiments. A similar single dose of 125 mg. in the form of an intravenous (or even intramuscular) aqueous solution would certainly be fatal.

Although Tubadil is never to be injected intravenously in clinical use, the question has arisen as to accidental intravenous injection of some of the Tubadil during the course of the intramuscular injection. In the above-described rabbit experiments the toxicity of intravenous Tubadil was only about one-third that of intravenous aqueous tubocurarine. Based on the rabbit comparison, it would appear that even if an entire 1 cc. of Tubadil (25 mg. of tubocurarine chloride) were to go into the vein, the toxicity would only be equivalent to that of about 8 mg. of intravenous aqueous tubocurarine, which is within the usual therapeutic range of the latter as used in surgery.

SUMMARY

1. Aqueous tubocurarine and repository tubocurarine (Tubadil) were compared for muscle relaxant effectiveness and toxicity in rabbits.

2. As compared by lethal dose with intravenous aqueous tubocurarine, intramuscular aqueous tubocurarine was one-half as toxic, intravenous Tubadil one-third as toxic, and intramuscular Tubadil only one-tenth as toxic.

3. At lethal dosages the overcurarization caused deaths very quickly with intravenous aqueous tubocurarine; whereas the deaths from intramuscular Tubadil were delayed, occurring after a prolonged period of gradually deepening respiratory depression.

4. At nonlethal levels and with the same degree of safety, the skeletal muscle relaxation achieved with intramuscular Tubadil was 8 times as long as with intramuscular aqueous tubocurarine and 30 times as long as with intravenous aqueous tubocurarine.

5. Overcurarization with Tubadil was counteracted by Tensilon or neostigmine, the longer-acting neostigmine appearing more effective.

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Ophthalmic Vehicles: The Effect of Methylcellulose on the Penetration of Homatropine Hydrobromide Through the Cornea*

By WILLIAM H. MUELLER† and DWIGHT L. DEARDORFF

The therapeutic effectiveness of a drug may be profoundly influenced by the vehicle in which it is administered. This is especially true of ophthalmic medication. Three formulations were investigated on nine individuals, aged 15–30, to determine their effectiveness as aqueous ophthalmic vehicles for homatropine hydrobromide. They were identical in electrolyte content, preservative content, and *p*H, but differed in methylcellulose content. They contained 1 per cent methylcellulose, 0.1 per cent methylcellulose, or no methylcellulose. Homatropine hydrobromide administered in 1 per cent methylcellulose induced greater cycloplegia and mydriasis at almost all doses. It is believed that the greater effectiveness is probably a result of increased viscosity rather than a lowering of surface tension caused by the methylcellulose. The methylcellulose reduced reflex lachrymation and caused no eye irritation or damage. The technique used provides a simple efficient method for further development and testing of new ophthalmic vehicles.

IN CLINICAL PRACTICE it is often desirable to obtain high therapeutic levels of drugs in the eye. This is done by administering drugs directly into the eye, orally, parenterally, or a combination of these modes of administration. The eye attempts to maintain its integrity and in many instances, depending upon the nature of the drug being administered, resists penetration of the drug through the blood aqueous barrier and through the corneal surface thus preventing the attainment of therapeutically effective concentrations of the drug.

Efficient administration of a drug allows one to attain therapeutic dosage levels, use smaller doses thus minimizing side effects, reduce the cost of treatment, lengthen the interval between treatments, and, in general, increase the comfort of the patient with a minimum of effort.

For the above reasons, studies have been made of the factors that affect the penetration of drugs through the cornea. Numerous factors have been isolated and much has been done to explain their part in the overall problem of corneal penetration. Some of the most important physicochemical properties of aqueous ophthalmic vehicles that influence corneal penetration are *p*H, isotonicity, surface tension, viscosity, miscibility with secretions of the eye, the relative affinity which the drug exhibits between vehicle and tissue fluids of the eye, and the water-lipid solubility partition coefficient of the drug.

In 1945, Swan (1) introduced to ophthalmology the use of methylcellulose as an agent to increase the viscosity of aqueous ophthalmic solutions. Methylcellulose is a cellulosic ether, soluble in water, and is available as a pure and uniform product. It is believed that increasing the viscosity of an aqueous ophthalmic solution probably causes an increase in contact time with the cornea thus enhancing the penetration of a drug if it is readily available from the solution in which it is administered. Swan also observed that methylcellulose did not appear to be absorbed from the conjunctival sacs of rabbits. Hydrophilic colloids, such as methylcellulose, whether of natural or synthetic origin, (2) have been known to adsorb drugs and retard their absorption.

Since the first introduction of methylcellulose to the field of clinical ophthalmology several recommendations (3, 4) have been made by clinicians as to the effectiveness of methylcellulose as a vehicle. However, little, if any, quantitative data has been presented to corroborate these clinical observations. The purpose of this report is to investigate the effectiveness of methylcellulose as an ophthalmic vehicle under clinical conditions.

Ophthalmic medications are usually introduced into the eye by placing them in contact with the conjunctiva and cornea. This is usually accomplished by introducing the vehicle into the upper or lower cul-de-sac or directly onto the surface of the cornea. The conjunctiva is a

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It is also adapted from the manuscript submitted by William H. Mueller which received honorable mention in the 1955 Lunsford Richardson Pharmacy Awards competition.
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mucous membrane consisting of a surface epithelium and underlying connective tissue (5). The cornea has a surface of stratified squamous epithelium without keratinization. Both the cornea and the conjunctiva are bathed in a precorneal film (6) which is secreted by the lacrimal and conjunctival glands. The composition of this film is kept very constant by a balance between secretion, evaporation, and drainage of the film solution. The film acts to lubricate, carry away desquamated cellular tissue, maintain a constant environment, and dilute any foreign substance such as therapeutic agents that may be introduced into the eye.

The cornea, for purpose of studying its permeability to various substances, can be considered to have two distinct absorbing barriers, the epithelium and the stroma. Cogen and Swan (7–9) demonstrated that the epithelium presented a barrier to substances not soluble in fat and that the stroma is more permeable to water-soluble compounds. The epithelium contains 10 per cent fat by dry weight and the stroma 1 per cent fat by dry weight.

A review of the literature reveals a paucity of information concerning the subject of contact time of ophthalmic vehicles with the cornea. Klein (10) recorded the use of egg white in cocaine solutions to increase the contact time of the drug with the eye. His results showed that a 0.1 per cent cocaine solution with egg white was equivalent to a 0.4 per cent cocaine solution in water alone. This increased effect was attributed to increased contact time of the solution with the cornea. Since then tragacanth, acacia, and gelatin have been used in ophthalmic solutions with little success due to their chemical instability, high index of refraction, and excellent bacterial and mold growth supporting qualities.

Swan and others (1, 3, 4, 6) have used methylcellulose clinically as an agent to increase the viscosity of aqueous ophthalmic solutions. Methylcellulose has also been found to be useful in contact lens solutions (1, 6, 11, 12). Here it exhibited a wetting property to optical plastics, was lubricating, soothing, and allowed the wearer a longer period of time before fogging occurred.

Methylcellulose (13) is a long chain cellulosic polymer prepared by reacting purified cotton linters or wood pulp with caustic soda and methyl chloride. Methylcellulose is available in different viscosity types (depending on the length of the polymer) and has the following characteristics: (*a*) It is soluble in water and produces viscous solutions at low concentrations. (*b*) It lowers the surface tension of aqueous solutions to 48–52 dynes/cm. in very low concentrations. (*c*) Solutions have a neutral *p*H and do not interfere with the buffering capacity of inorganic materials. (*d*) Solutions are stable from *p*H 2 to 12. (*e*) Solutions are stable to autoclaving procedures. (*f*) The refractive index of 2 per cent solutions at 20° is 1.3355. (This does not interfere with normal vision.) (*g*) Solutions do not support mold growth under ordinary conditions of usage. (*h*) It is less soluble in hot water than in cold.

Homatropine HBr was selected as the drug with which to study penetration through the cornea in the presence and absence of methylcellulose in aqueous solutions. Homatropine causes both cycloplegia and mydriasis in humans, 2–5 per cent solutions are commonly used, complete mydriasis is reached in one-half hour and lasts a maximum of two days. Maximum cycloplegia is reached after one hour and complete recovery occurs after one and one-half to two days (14, 15).

A number of methods are available for measuring the degree of cycloplegia (2, 16–19). Some of these (16) are very accurate and concise, but require special equipment and much time to perform. The method selected for these experiments utilized the "near point" principle embodied in a device called the "Prince Rule" (American Optical Co.) (17). The Prince Rule is essentially a meter stick upon which a sliding objective card has been placed. The subject places one end of the rule against the cheek below one eye while the other eye is covered. He then moves the objective card close to the eye until the figures just become blurred. This represents the near point of vision and can be readily converted to diopters of accommodation.

The amount of accommodation that a person has decreases with increased age (18, 20). For example, 15 diopters at 12 years, 6.5 diopters at 30 years, and one diopter at sixty years of age. For this reason, subjects as young as were available were selected for the experiments performed. The amount of accommodation in each eye of a subject is generally expected to be the same.

Mydriasis was measured by comparing the diameter of the pupil with a series of holes drilled in a plastic disk. The diameter of these holes ranged from 2.5 to 9.0 mm in 0.5 mm graduations.

EXPERIMENTAL

Preparation of Solutions.—Three basic formulations were investigated. Their composition varied only in methylcellulose content. The formula and

certain physical properties of these three preparations are presented in Table I.

TABLE I

	Per Cent Composition		
	A	B	C (Control)
Formula			
Sodium acid phosphate (anhydrous) U. S. P.	0.40	0.40	0.40
Disodium phosphate (anhydrous) U. S. P.	0.47	0.47	0.47
Methylparaben U. S. P.	0.10	0.10	0.10
Methylcellulose 4000 c. p. s. N. F.	1.0	0.10	...
Distilled water to make	100 cc.	100 cc.	100 cc.
Physical properties			
*p*H	6.6	6.6	6.6
Viscosity (c. p. s. at 35°)	230–235[b]	12–15[b]	Less than 1[a]
Surface Tension (at 25°)[c]	48–52	49.3	69.7

[a] Literature data. [b] Brookfield Model RVT viscometer. [c] Cenco du Nouy tensiometer.

The following procedure was used to prepare the experimental solutions: (*a*) The phosphate buffer salts and preservatives were dissolved in about 80% of the water with agitation.

(*b*) This solution was heated to 80–90° and the methylcellulose added with good agitation. With continued stirring, the solution was cooled to room temperature and the remainder of the water added.

(*c*) The solution was placed in 20 cc. parenteral-type vials and stoppered with rubber stoppers and aluminum seals.

(*d*) The solutions were autoclaved at 15 lb. steam pressure for 20 minutes.

(*e*) Homatropine HBr U. S. P. was added as needed to the vials of sterile solution just prior to administration of the solution.

Using this technique, it was possible to administer essentially sterile and always freshly prepared solutions of homatropine hydrobromide. The only nonsterile material used in the procedure was the homatropine. The container from which the homatropine was taken was opened just prior to the experiment being performed and was reserved exclusively for this study to prevent contamination of the material.

One per cent and 0.1% concentrations of methylcellulose were investigated for several reasons. Methylcellulose 4000 c. p. s. has been used clinically (1, 3, 4, 6) and is used in commercial ophthalmic preparations in concentrations ranging from 0.5 to 1.0%. In this concentration range, the solutions are viscous, but can be handled in a syringe or medicine dropper. At concentrations above 1.0% methylcellulose 4000 c. p. s. solutions become thixotropic and plastic and cannot be handled conveniently unless administered from a collapsible ointment tube. For these reasons, 1% methylcellulose was selected as a practical formula concentration.

Laboratory experiments showed that methylcellulose 4000 c. p. s. lowered surface tension to 48–52 dynes/cm. even at concentrations as low as 0.01% in aqueous solution. It is well known that lowered surface tension greatly increases the absorption of certain drugs from ophthalmic solutions (9, 21–24). In order to resolve the question as to whether surface tension or viscosity may be responsible for increased absorption of homatropine from aqueous solution, a solution containing 0.1% was selected to investigate the lowered surface tension phenomena. It was hypothesized that a 0.1% solution even when diluted with tear fluids would still lower surface tension to the maximum and not be so viscous as the 1% methylcellulose solution.

Methyl parahydroxybenzoic acid was selected as preservative for use in these experiments for several reasons. Aside from its excellent preservative properties (25, 26) methyl parahydroxybenzoic acid was known not to influence the penetration of drugs through the cornea (27). This was an important factor to control as other preservatives, such as certain of the quaternary ammonium compounds (9, 22, 27), have been shown to enhance the penetration of drugs through the cornea by lowering the surface tension of the solution.

General Experimental Procedure.—*Selection of Dose Volume and Concentration of Homatropine.*—Preliminary experiments indicated that 0.1 cc. was well tolerated by the average lower cul-de-sac. At this dose volume, solutions of homatropine in concentrations ranging from 0.1 to 1% induced adequate pharmacologic responses in human subjects for experimental procedures.

Selection of Experimental Subjects.—Caucasian subjects between the ages of 15 and 30 years were used for this investigation. This age group was used to insure an adequate amount of accommodation. Subjects who wore glasses wore them throughout the tests. The subjects were also selected because of their ability to follow directions and reproduce accurately near-point readings under experimental conditions.

Experimental Procedure.—(*a*) Five near-point determinations were made on each eye of the subject. The pupil diameters were then measured using the plastic disk.

(*b*) Using a sterile 1-cc. tuberculin syringe (without needle), 0.1 cc. of an experimental solution was introduced in the lower cul-de-sac of one eye. The process was repeated with the other eye, using a different experimental solution. The subject kept both eyes closed for one minute after instillation of the solution.

(*c*) Near-point and pupil-diameter readings were repeated at 45-, 60-, and 90-minute intervals in all experiments. Two-hour and 24-hour readings were obtained whenever possible.

Calculations.—Table II includes all the data taken in one experiment on one subject. The column "Left Near Point in cm." contains the near point of vision distances recorded in centimeters from the left eye. The column "Left Near Point Average in cm." is the mean of the five near-point readings in cm. The column "Left Eye Diopters" is the average near-point distance converted to diopters. By definition, the refracting power of a

lens is expressed as the reciprocal of the focal length given in meters and the unit is called a diopter. Therefore, using the data from Table II:

$$1.0 \text{ meter}/0.114 \text{ meter} = 8.8 \text{ diopters.}$$

The figures in the column "Left Eye % Accommodation" were obtained by assuming the initial dioptric reading to be 100% accommodation. The 76% accommodative value appearing for the left eye at the 45-minute time interval was obtained as follows:

$$\frac{\text{New diopters}}{\text{Initial diopters}} = \frac{6.7 \times 100}{8.8} = 76\% \text{ Accommodation}$$

Pupil diameter readings were recorded as they were obtained using the plastic disk.

Experimental Design.—The primary objective of the study was to determine if a methylcellulose solution of homatropine HBr was more effective pharmacologically than was an aqueous solution of the same drug. To accomplish this, three groups of experiments were performed.

Threshold Determining Experiments.—On four different experimental days, each subject was administered 0.1 cc. of 0.25, 0.50, 0.75, and 1.0% homatropine solutions. The drug was administered to one eye in the aqueous control solution and to the other eye in the 1% methylcellulose vehicle. This series of doses was given to establish a threshold at which differences in performance could be observed.

Control and experimental solutions were alternated between eyes for each experiment. At least one week was allowed to elapse between successive experiments on an individual.

Replicate Experiments.—After a threshold was established for each subject, the experiment was repeated to verify its validity.

Viscosity vs. Surface Tension Experiments.—Methylcellulose in solution acts to lower surface tension at all concentrations and to increase viscosity at higher concentrations such as 1.0%. The effectiveness of 1.0% and 0.1% solutions of methylcellulose was investigated using the threshold dose of homatropine derived from the screening experiments.

RESULTS

Threshold Determining Experiments.—Figure 1 and Table II illustrate the loss of accommodation and the mydriasis that occurred after administering 0.25% doses of homatropine to one subject in aqueous and methylcellulose vehicles. Figure 1 demonstrates the relationship of cycloplegia and mydriasis when plotted against the same time abscissa. Table II includes the five near-point readings made at each time interval. It can be seen in this illustration that little pharmocologic response resulted from a dose of 0.25% homatropine in aqueous solution. However, considerable pharmacologic response occurred from the same dose of homatropine when administered in a 1% methylcellulose vehicle. Figure 2 illustrates the loss of accommodation that occurred when graded doses of homatropine were administered. At doses above 0.25% a considerable response was obtained from

TABLE II.—CYCLOPLEGIC AND MYDRIATIC RESPONSE TO A DOSE OF 0.25% HOMATROPINE HYDROBROMIDE IN AQUEOUS AND 1% METHYLCELLULOSE SOLUTIONS

	Subject's Age: 22					Eye Color: Other Than Brown				
Time Interval, Minutes	Left Near Point, cm.	Left Near Point, Av.	Left Eye, Diopters	Left Eye, % Acc.	Left Pupil Diam., mm.	Right Near Point, cm.	Right Near Point, Av.	Right Eye, Diopters	Right Eye, % Acc.	Right Pupil Diam., mm.
	Dose: 0.1 cc. of 25% Homatropine, 1.0% Methylcellulose in Right Eye									
0	12.1	11.7	8.5	100	3.0	11.4	11.4	8.8	100	3.0
	11.0					11.3				
	11.8					10.7				
	11.7					12.2				
	11.7					11.6				
45 Min.	10.5	10.7	9.3	109	3.5	16.2	14.8	6.7	76	3.0
	11.1					14.8				
	10.7					14.1				
	10.6					13.9				
	10.8					14.1				
60 Min.	11.8	11.3	8.9	105	4.0	18.5	20.2	4.9	56	6.0
	10.3					21.2				
	11.1					21.6				
	11.7					20.2				
	11.4					21.2				
90 Min.	10.7	11.2	8.9	105	3.5	22.6	20.7	4.8	54	6.5
	11.1					20.3				
	11.0					19.9				
	11.5					19.1				
	11.5					19.4				
24 Hr.	11.0	11.6	8.6	101	5.5	12.3	13.0	7.7	87	4.5
	11.3					13.5				
	12.5					14.2				
	11.4					12.5				
	11.6					12.4				

both vehicles at each dose level. The threshold dose for this subject was established at 0.25% homatropine. The data used to prepare Fig. 2 are presented in Table III.

A threshold was established for eight of the nine subjects tested. Six subjects exhibited a threshold at a dose of 0.25% homatropine and in two subjects, a threshold was established at 0.50%. The irides of the latter two subjects were brown.

The threshold herein referred to is that dose at which a minimum of pharmacologic response resulted from the aqueous solution while a substantial response was obtained from the 1% methylcellulose solution. It is also the dose at which the greatest difference in response between the two treatments usually occurred.

Replicate Experiments at Threshold Dose.—In those subjects in which a threshold was determined, replicate experiments were performed. The results confirmed the original experimental data.

Comparison of 1% and 0.1% Methylcellulose at Threshold Doses.—It was found as shown in Fig. 3 that the pharmacologic response obtained from homatropine in 0.1% methylcellulose was of the same magnitude as that obtained when the drug was administered in the aqueous vehicle (Figs. 1 and 2).

The subjective responses of the subjects were solicited upon instillation of the solutions. In most cases, the aqueous solution caused a slight burning sensation for one to two minutes after instillation whereas the methylcellulose solution was considered to be soothing by comparison and in some instances painless. It was noted that less reflex lachrymation and squinting occurred in the eyes in which the methylcellulose solutions were introduced. No incidence of eye damage or irritation occurred during the course of these experiments.

In a few subjects, a slight white crust appeared along the lid margins the day after the experiment in the eye in which the 1% methylcellulose was administered. The crust was easily removed with water, and, in most instances, was unnoticed by the subject.

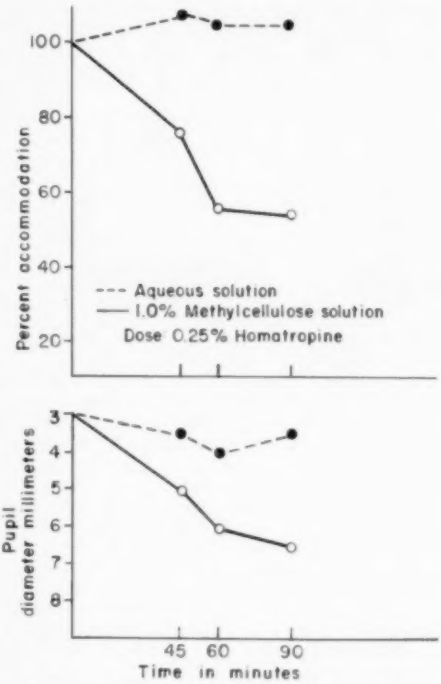

Fig. 1.—Cycloplegic and mydriatic response to 0.25% homatropine in aqueous and 1% methylcellulose solutions.

DISCUSSION

In previous ophthalmic research problems performed by Crowell (28) and Southard (29) of this laboratory, attempts were made to determine the effectiveness of various alkaloids and synthetic drugs upon the eyes of intact rabbits when administered in a variety of vehicles. Unless one works with the excised cornea or performs such techniques as a limbal puncture (7–9, 30), pupil response is the only pharmacologic attribute that can be measured in rabbits when using mydriatics or miotics. As Floyd, Kronfeld, and McDonald (31) noted, the performance of clinical tests on human eyes offers an opportunity to collect data which are of much more interest and significance than those obtained from laboratory animals. For this reason, it was decided to use human subjects and select a drug that would affect several measurable responses. Homatropine hydrobromide was selected as the drug to be used in these experiments because it acts both as a cycloplegic and mydriatic in humans and has a relatively short period of activity in the tissues (15).

The threshold tests in which each subject was given doses of homatropine ranging from 0.25–1% in aqueous and 1% methylcellulose vehicles served two purposes. First, a threshold was established for each subject at which a difference in response could be observed. Secondly, it allowed the investigator to determine which subjects were suitable for this type of testing in which the subjective near point response of the subject had to be relied upon to obtain the data.

Threshold experiments were performed on nine subjects. The results obtained from eight of these demonstrated that a type of threshold could be recognized as a result of administering graded doses of homatropine in aqueous and 1% methylcellulose vehicles. One subject gave erratic responses to the screening experiments and for this reason no replicate experiments could be performed on this subject.

In two subjects, a threshold was established at 0.5% homatropine. Both of these subjects had brown eyes. In six subjects, with eyes other than brown, a threshold was established at 0.25% homatropine. Although large differences in response were observed between the two treatments at doses above the threshold doses, the lowest dose at which a substantial difference in response occurred was selected as the threshold dose to inconvenience the experimental subjects as little as possible in later experiments, and also to be able to detect smaller differences in the effect produced by adding methylcellulose to the solution. A high concentration of

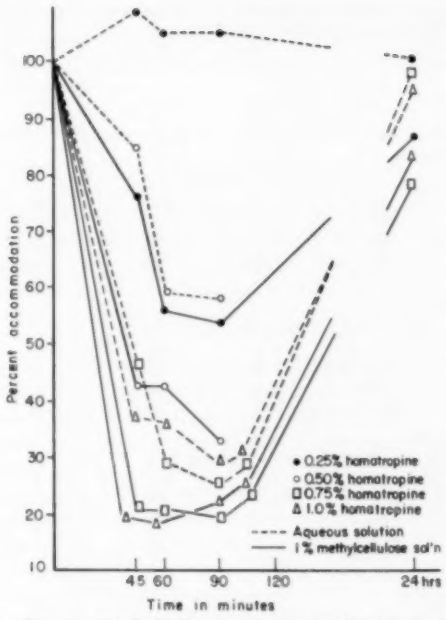

Fig. 2.—Cycloplegic response to graded doses of homatropine in aqueous and 1% methylcellulose solutions.

homatropine would give a maximal effect regardless of the vehicle. Greater cycloplegic and mydriatic responses were obtained from the 1% methylcellulose vehicle at all concentrations of homatropine in almost every experiment. This, in itself, is additional encouraging information as to the effectiveness of 1% methylcellulose as an aqueous vehicle. During the course of the screening tests, one reversal took place in which the aqueous solutions of homatropine gave a better pharmacologic response than did the homatropine in the methylcellulose vehicle.

The variation and unusual responses which occurred during the testing could have been attributed to several causes. The amount of accommodation that each subject exhibited varied very slightly (perhaps one diopter maximum) between testing days. This variation might have been a result of the amount of rest the subject had had, the amount of strain the eye had recently been subjected to, and the mood of the subject. However, good replication was obtained in seven of eight subjects.

The experimental variable which was most difficult to control and which probably contributed most to experimental error was the actual administration of the solutions. Unlike many pharmacologic experiments, in which a calculated dose based on body weight can be administered parenterally without loss of the drug, administration of ophthalmic drugs onto the surface of the cornea presents a very precarious situation because of its exposed position in the palpebral fissure. When administering solutions onto the mucous membrane of the eye, it is usually placed in the upper or lower cul-de-sacs which are only potential sacs until their walls are distended by some force. In the experiments herein reported, the solutions were administered into the lower cul-de-sac. Considerable difference was observed between subjects in the potential volume of the sac and tension under which it was held. This could have been a contributing factor to the problem of administering the drug quantitatively to the eye.

Figure 3 shows the results of administering threshold doses of homatropine in 1% and 0.1% methylcellulose vehicles. The results of the tests with 0.1% methylcellulose are substantially the same as those obtained with aqueous vehicles. This seems to indicate that increased contact time rather than lowered surface tension is responsible for the increased response from 1% methylcellulose solutions.

The following hypotheses are set forth as possible explanations of the differences observed between aqueous and 1% methylcellulose solutions as a vehicle for homatropine:

(*a*) The surface of the eye is continually being bathed with tear fluids. The secretion and removal of these fluids by drainage and evaporation represent a dynamic condition.

(*b*) When foreign substances such as eye drops or ointments are introduced onto the surface of the eye, the eye reflexly increases the rate of lachrymation.

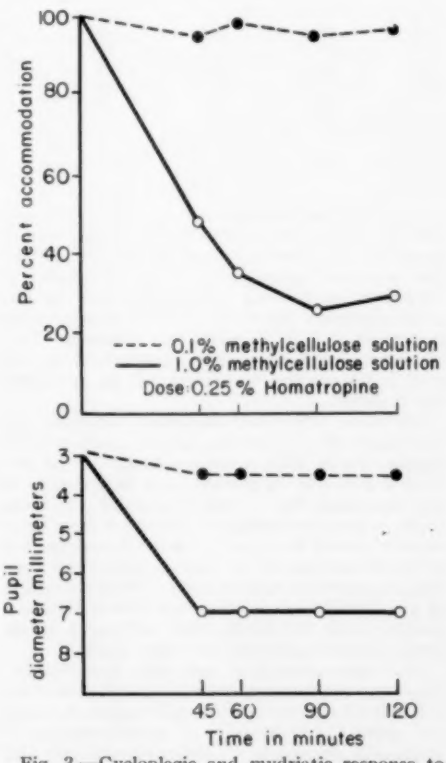

Fig. 3.—Cycloplegic and mydriatic response to 0.25% homatropine in 0.1% and 1.0% methylcellulose solutions.

TABLE III.—CYCLOPLEGIC AND MYDRIATIC RESPONSE TO A SERIES OF DOSES OF HOMATROPINE HYDROBROMIDE IN AQUEOUS AND 1% METHYLCELLULOSE SOLUTIONS

	Subject's Age: 22				Eye Color: Other Than Brown			
Time Interval, Minutes	Left Near Point, Av. cm.	Left Eye, Diopters	Left Eye, % Acc.	Left Pupil Diam., mm.	Right Near Point, Av. cm.	Right Eye, Diopters	Right Eye, % Acc.	Right Pupil Diam., mm
				Experiment A				
	Dose: 0.1 cc. of 0.25% Homatropine, 1.0% Methylcellulose in Right Eye							
0	11.7	8.5	100	3.0	11.4	8.8	100	3.0
45	10.7	9.3	109	3.5	14.8	6.7	76	5.0
60	11.3	8.9	105	4.0	20.2	4.9	56	6.0
90	11.2	8.9	105	3.5	20.7	4.8	54	6.5
24 Hr.	11.6	8.6	101	5.5	13.0	7.7	87	4.5
				Experiment B				
	Dose: 0.1 cc. of 0.5% Homatropine, 1.0% Methylcellulose in Right Eye							
0	10.1	9.9	100	3.0	10.2	9.8	100	3.0
45	11.9	8.4	85	3.5	23.8	4.2	43	6.5
60	17.0	5.9	59	5.0	23.5	4.2	43	7.5
90	17.2	5.8	58	5.0	30.9	3.2	33	7.5
				Experiment C				
	Dose: 0.1 cc. of 0.75% Homatropine, 1.0% Methylcellulose in Left Eye							
0	11.4	8.8	100	3.5	11.8	8.5	100	3.5
45	51.8	1.9	21	7.5	25.7	3.9	46	5.0
60	52.9	1.9	21	7.5	40.5	2.5	29	5.0
90	56.2	1.8	20	7.5	44.7	2.2	26	5.5
105	48.0	2.1	24	7.0	39.9	2.5	29	6.0
24 Hr.	14.2	7.0	79	6.0	11.7	8.5	100	5.0
				Experiment D				
	Dose: 0.1 cc. of 1.0% Homatropine, 1.0% Methylcellulose in Right Eye							
0	10.9	9.2	100	3.5	10.9	9.2	100	3.5
45	29.1	3.4	37	6.0	53.2	1.9	21	7.0
60	30.2	3.3	36	6.5	50.1	2.1	22	7.5
90	36.8	2.7	29	7.0	50.9	2.0	22	7.5
105	36.7	2.7	29	6.5	44.7	2.2	24	7.0
24 Hr.	11.1	9.0	98	5.5	12.5	8.0	87	6.5

(*c*) When aqueous solutions are introduced into the eye, the solution is rapidly diluted and washed from the eye allowing only a short period of time for absorption through the cornea. According to Swan (6), maintaining the lubricity and viscosity of the precorneal film is as important as maintaining isotonicity and *p*H in ophthalmic solutions. This, of course, is not done when aqueous solutions are used in the eye and the integrity of the precorneal film is altered.

(*d*) When 1% methylcellulose solutions are introduced into the eye they spread evenly over the surface of the globe, impart viscosity to the precorneal film, and, in general, alter the integrity of the precorneal film a minimal amount. The viscosity of the methylcellulose prevents it from being rapidly washed from the eye and the maintenance of conditions close to the normal physiologic state prevents excessive lachrymation. The combination of these two factors increases the contact time of a solution with the cornea thus allowing a longer period of time for absorption to take place.

The observation that less reflex lachrymation, squinting, and pain occurred upon instillation of the 1% methylcellulose solutions is in agreement with the general acceptability of methylcellulose in ophthalmic preparations. Other investigators (12, 32) have reported on the usefulness of methylcellulose in contact lens solutions and for the replacement of deficient lachrymal secretions, (33, 34).

In this work only homatropine hydrobromide has been used, but because of the similarity in structure between homatropine and other tropic acid derivatives, methylcellulose will probably enhance the absorption of these drugs, also. It may be possible to determine by spectrophotometric or dialysis techniques if methylcellulose adsorbs drugs in solution (35–37). In this way, many ophthalmic drugs could be screened *in vitro* to determine the most efficient and effective vehicle for their administration.

SUMMARY

1. Three basic formulations were investigated to determine their effectiveness as aqueous ophthalmic vehicles for homatropine hydrobromide. The three formulations were identical in electrolyte content, preservative content, and *p*H but differed in methylcellulose content, surface tension or viscosity. Solution A contained one per cent methylcellulose 4000 c. p. s., solution B contained 0.10 per cent methylcellulose 4000 c. p. s. and solution C contained no methylcellu-

lose. It was found that homatropine administered in a one per cent methylcellulose vehicle induced greater cycloplegia and mydriasis at almost all doses.

2. Subjects were screened to establish a threshold by giving them a range of doses of homatropine hydrobromide in aqueous and 1% methylcellulose solutions. Of the nine subjects tested a definite threshold was established in eight. Those having eyes other colors than brown exhibited a threshold at 0.25 per cent homatropine. The two subjects having brown eyes exhibited a threshold at 0.50 per cent homatropine.

3. The increased pharmacologic response obtained from homatropine in one per cent methylcellulose solution was probably due to increased contact time with the eye caused by the viscous nature of the vehicle. Both one per cent and 0.1 per cent methylcellulose in aqueous solution lower surface tension to 48–52 dynes/cm. However, the viscosity of these two solutions is substantially different, the one per cent solution being much more viscous. When evaluated as vehicles for homatropine, the performance of the 0.1 per cent solution was comparable to that of an aqueous vehicle showing no increased absorption.

4. Less reflex lachrymation occurred from the instillation of methylcellulose solutions than from the aqueous vehicle. Decreased reflex lachrymation further aids in increasing contact time with the cornea.

5. No incidence of eye irritation or damage occurred during the course of the experiments indicating the acceptability of methylcellulose as a component of certain ophthalmic solutions.

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Toxicity, Skin Irritant Properties and Absorption of N-*n*-Butyl-3-Phenylsalicylamide*

By V. P. SEEBERG, JOHN HIDALGO, WERNER WILKEN, H. N. BENIAMS, and JOHN LUNDBLAD

N-*n*-Butyl-3-phenylsalicylamide, a new potential antifungal agent, was found to possess a low order of systemic toxicity. No evidence of topical irritation or toxicity could be evoked by repeated application or relatively large amounts of the compound to young rabbits. The compound is only slightly absorbed. By administration in a special vehicle, absorption of approximately 2 per cent of an oral dose was obtained, which allowed the establishment of a tissue distribution pattern for the drug.

FROM A SERIES of substituted salicylamides synthesized by Jules, *et al.* (1), and screened for antifungal activity by Hok and Pilcher (2), N-*n*-butyl-3-phenylsalicylamide (Compound S.2-339) emerged as the most potent compound in the group in its activity against *Trichophyton mentagrophytes*. Inhibition of growth of this organism was brought about by a concentration of 1:512,000 in Sabouraud's agar and 1:64,000 in the presence of serum. The compound also showed a high order of activity against *Microsporum audouini*, the causative agent of tinea capitis (ringworm of the scalp). Salicylic acid is known to be mildly irritating and salicylanilide must be restricted in its use as a topical antifungal agent (3) because of potential irritant effects on the skin. It was, therefore, deemed necessary to establish some parameters of toxicity and skin irritant properties of this related compound in animals before investigating its possible value as a therapeutic agent in man. Because of the low order of toxicity found, the possibility of achieving systemic antifungal concentrations of the compound was also investigated. This report is concerned with the results of studies on these points.

EXPERIMENTAL

For oral and parenteral administration, suspensions of the compound were made in 1% tragacanth solution. The compound, which occurs in the form of colorless, nonhygroscopic needles, was reduced to a 100-mesh powder before incorporation. Most suspensions were 4% in concentration, but in some of the high oral doses, concentrations as high as 32% were used. Mice of the Webster Swiss strain and Sprague-Dawley rats were used in the acute studies.

Subacute toxicity studies were performed on immature Sherman Wistar rats. All animals were observed for a preliminary two-week period to assure normalcy and adequate weight gain on a normal diet. The compound was mixed intimately with a minimum weight of diet based on the total weight of the experimental groups, so that all of the diet would be ingested. When all of the diet containing the compound had been consumed, the animals received additional food *ad libitum*. This procedure was repeated daily for ninety days. At the end of thirty days, two males and two females from each group under observation were sacrificed and the organs examined for gross pathology and organ weights were determined on the heart, adrenals, spleen, kidneys, lungs, and liver. The stomach, intestine, pancreas, heart, spleen, lung, liver, kidney, gonads, and brain were examined microscopically. The heart blood from the sacrificed animals was used for sedimentation rate, coagulation time, hematocrit, and prothrombin time determinations. Tail blood was drawn from all the surviving animals and used for red cell count, white cell count, differential count, and hemoglobin determinations. At the end of ninety days all the surviving animals were weighed and sacrificed and the organs examined for gross pathology. Organ weight determinations and microscopic examination of tissues were performed on the same organs as listed for the thirty-day observation. At the ninety-day observation, hematology was performed on heart blood from all the animals.

Skin irritation and local toxicity tests were performed on young albino rabbits, carefully selected for smoothness and softness of skin texture. In the small area tests the compound was applied in the form of an ointment to marked-off areas of one square inch to each of the test animals at the rate of 100 mg. per square inch daily, five days a week for two weeks. Contact was for eight hours a day. The animals were placed in stocks in the morning at the time of application and remained there during the eight-hour period, at the end of which any unabsorbed ointment was removed and the animal was then released until the following day. Irritation scoring was made by the method of Draize, *et al.* (4). For the large area irritation and local toxicity experiment, the rabbits were first subjected to a hematological examination including complete blood cell counts and determinations of blood clotting times, hematocrit, prothrombin time, and sedimentation rate. Also, a qualitative sugar and albumin test was applied to the urines. The rabbits were then carefully shaved of hair on the dorsal and lateral surfaces representing approximately 20% of the total body surface area and anointed over these areas daily, in the morning with 4 Gm. per Kg. of an ointment of the compound. The ointment base was a Carbowax-polyethylene glycol formulation intended for use clinically. After application, the

* Received October 8, 1955, from the Research Division, Cutter Laboratories, Berkeley, Calif.

animals were immobilized in stocks for the ensuing eight-hour period to prevent licking and rubbing off of the ointment. The animals were then returned to their cages for the night. This treatment was repeated daily, five days a week, for four weeks. Throughout the experimental period the animals were observed for any evidence of irritation at the site of application and for any other signs or symptoms of either local or systemic toxicity. At the end of the application period the hematological tests and urine tests run at the beginning of the experiment were repeated. The animals were then sacrificed and subjected to gross and microscopic examination of organs and tissues.

Methods for analysis of blood and tissue were based on the finding that dilute solutions of the compound in water and various organic solvents emit a strong fluorescence upon illumination with light from a monochromator at wavelengths below 3700 Å. Levels of 10 γ per cc. and higher in serum or body fluids could be read directly with a Brice light scattering photometer converted to a sensitive fluorophotometer by the insertion of a Pfaltz-Bauer UV limiting filter at the light source and a Pfaltz-Bauer UV eliminating filter no. 038 at the photomultiplier. A 1:100 dilution of the sample in 0.01 *N* NaOH was first made in order to reduce interference by extraneous material to a minimum. The instrument was then set to give a galvanometer reading of 100 against 0.10 γ per cc. of the compound in 0.01 *N* NaOH, and further dilutions of the unknown were made if necessary to give a reading in the upper end of the scale. For samples of serum or tissue with levels below 10 γ per cc. it was necessary to first extract with 10 volumes of 95% ethanol. Tissues were minced before this extraction procedure and the leaching process was carried out for twenty-four hours with occasional agitation. The supernatant was removed and diluted with sodium hydroxide solution as before to the proper concentration for the fluorometric reading. In all serum and tissue determinations, a blank was also read to allow a correction for normal fluorescence of the particular serum or tissue under study. In the case of serum, the blank was prepared from a sample of the animal's own blood drawn before drug administration. In the case of other body tissues, it was necessary to prepare representative blanks from corresponding tissues of another animal. Using the latter procedure, concentrations as low as 0.1 γ per Gm. of tissue could be detected.

RESULTS

Systemic Toxicity.—The LD_{50} of N-*n*-butyl-3-phenylsalicylamide by the intraperitoneal route was found to be 1.85 Gm. per Kg. in mice and 2.4 Gm. per Kg. in rats with 19/20 confidence limits of 1.6–2.2 and 2.1–2.8, respectively. Orally, it was administered to nine groups of mice in doses ranging from 100 mg. to 16 Gm. per Kg. The highest dose was the largest amount that could be administered without regurgitation. No mortalities or gross evidences of toxicity were observed in any of the groups. Rats also tolerated 16 Gm. per Kg. orally with no effects and rabbits were given up to 3.2 Gm. per Kg. without effect.

A subacute toxicity study was performed on three groups of rats each containing 10 males and 10 females. One group received 300 mg. per Kg. daily, the second received 1 Gm. per Kg. daily and the third group was maintained as a control. There were no significant differences in hematology, body weights, organ weights, or gross or microscopic appearance of tissues among the control or experimental groups at the end of thirty or ninety days which could be attributed to the experimental procedure.

Topical Irritation and Local Toxicity.—In the small area irritation tests the compound was tested in ointments of 2% and 10% concentration. At no time during the two-week experimental period did any of the marked-off areas show a scorable degree of irritation. The large area tests also failed to show any evidence of local irritation during the prolonged application of considerably larger amounts. A 5% ointment was used in the latter test, which in the amount applied, provided contact with 200 mg. of the compound per Kg. daily. The animals also appeared normal in other respects throughout the experimental period. No significant changes were seen in either the hematological or the urine tests run at the beginning and the end of the experimental period. All organs appeared normal upon gross examination at the time of autopsy, and microscopic examination of the treated skin and the principal organs did not reveal any local or systemic pathology.

Absorption and Distribution in Tissues.—Intramuscular injection of 100 mg. per Kg. as a 10% aqueous suspension to rabbits resulted in only traces of the compound in the serum, indicating little or no absorption from this site. Oral doses of 1 Gm. per Kg. administered as an aqueous suspension to fasted rabbits produced peak serum levels in the order of 1 γ per cc., which indicated that the compound was not appreciably absorbed from the gastrointestinal tract, and which in turn is the likely explanation for its very low toxicity by the oral route of administration. It was found, however, that oral absorption of the compound could be enhanced by administration as a 10% solution in a vehicle consisting of 25% Tween® 80, 25% propylene glycol, and 50% water. An oral dose of 500 mg. per Kg. of the compound in this vehicle to rabbits produced serum levels of 5 to 18 γ per cc. at ninety minutes, 10 to 14 γ per cc. at six hours and 0.7 to 2 γ per cc. sixteen hours after administration.

Two rabbits given 250 mg. per Kg. of the compound orally in the special vehicle described above were sacrificed ninety minutes later and the tissues analyzed. Tissue levels of the compound found are given in Table I.

TABLE I.—TISSUE LEVELS OF S.2-339 IN GAMMAS PER GM. OF TISSUE AFTER ORAL ADMINISTRATION OF 0.25 GM. PER KG. TO RABBITS IN A SPECIAL VEHICLE

Tissue	Rabbit #1	Rabbit #2
Skin	0.0	0.6
Muscle	0.3	0.3
Liver	10.0	9.0
Brain	0.3	1.0
Kidney	11.0	14.0
Serum	2.5	3.1
Urine	158.0	334.0

In examining the tissue levels in Table I, it should be noted that all levels are reported in gammas, and therefore the total recovery still represents only a very small portion of the administered dose. A rough summation of the compound found in the urine and tissues indicated that less than 2% of the compound administered could be accounted for. This was further confirmed by an analysis of the entire gastrointestinal contents of the test animals at the end of the absorption period. Essentially, 100% of the administered compound, within the limits of error of the assay, was recoverable from the gastrointestinal tract.

SUMMARY

1. N-*n*-Butyl-3-phenylsalicylamide has a low order of acute toxicity. The LD_{50} intraperitoneally was found to be 1.85 and 2.5 Gm. per Kg. for mice and rats, respectively. Mice and rats tolerate doses as high as 16 Gm. per Kg. orally without toxicity. Rabbits were given up to 3.2 Gm. per Kg. without effect.

2. Oral subacute administration of 300 mg. and 1 Gm. per Kg. daily to rats for ninety days did not result in any significant deviation from control animals in hematology, body weights, organ weights, or gross or microscopic appearance of tissues.

3. Small and large area irritation tests in rabbits did not reveal any skin irritant properties for the compound. Large amounts applied daily for one month in the large area tests did not cause any significant changes in hematology, urinalysis, or gross or microscopic appearance of tissues.

4. The compound is not appreciably absorbed from the gastrointestinal tract. When absorption was increased somewhat by a special solvent vehicle, highest concentrations of absorbed compound were found in the liver and kidney and lowest in muscle, brain, and skin. Serum concentrations were intermediate.

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A New Approach to the Assay of Alkaloidal Crude Drugs. II. Ipecac*

By EINAR BROCHMANN-HANSSEN

A new method is proposed for the assay of ipecac. The alkaloids are extracted, purified, and separated into phenolic and nonphenolic fractions by means of ion exchange resins. The method is simple and gives results which are appreciably higher than those obtained by the official procedure.

IPECAC CONTAINS at least five alkaloids. Two of these are phenols, cephaeline and psychotrine; the others are nonphenolic in nature, emetine, *o*-methylpsychotrine, and emetamine (1). Emetine and cephaeline occur in the largest amounts. The two main varieties of ipecac, both of which are official in U. S. P. XV, are Rio ipecac (*Cephaelis ipecacuanha* Rich.) and Cartagena or Nicaragua ipecac (*Cephaelis acuminata* Karsten). There is not a great deal of difference in the total alkaloid content of these two varieties, but the Nicaraguan variety has a much higher percentage of the less valuable cephaeline (1). Some pharmacopeias analyze the drug for nonphenolic alkaloids as well as for total alkaloids. According to U. S. P. XV, ipecac must contain not less than 2 per cent of ether-soluble alkaloids. This expression stems from the assumption that psychotrine is not extracted in the official assay procedure, since it is much less soluble in ether than the other ipecac alkaloids (2–4). However, when freshly precipitated, psychotrine is quite soluble in ether (5). Unpublished work carried out in this laboratory shows that psychotrine is present in the ether extract obtained by the method of the U. S. P. Therefore, if the official assay does not yield the total alkaloid content of ipecac, this is due to the limitations inherent in the maceration process used for extraction.

In two previous communications, the author has shown that an alkaloidal crude drug can be extracted by being shaken with an aqueous sus-

* Received February 13, 1956, from the University of California School of Pharmacy, San Francisco, Calif.

pension of a cationic exchange resin (6, 7). The alkaloids are rapidly taken up by the resin in exchange for hydrogen ions, but can be recovered quantitatively by elution with alcoholic ammonia. Many impurities are also adsorbed on the resin and eluted together with the alkaloids. These impurities can be removed by allowing the eluate to flow through a porous anionic exchange resin. This resin will adsorb the colored impurities, but will let the alkaloids pass through unhindered provided they are nonamphoteric in nature. Amphoteric alkaloids will be retained on the anionic resin, but can be recovered by elution with acid (7). By these simple ion exchange techniques, it becomes possible to extract the total alkaloids from the crude drug, to purify them, and to separate them into amphoteric and nonamphoteric fractions.

In trying to develop a method for the assay of ipecac along the lines indicated above, experiments were first carried out with pure samples of emetine and cephaeline.[1]

cephaeline hydrochloride was passed through a column of Dowex 1-X_1, without previous exchange on Dowex 50-X_2. The results of these experiments are given in Table I.

Quantitative Determination of Ipecac Alkaloids.—Ultraviolet spectrophotometry provides the simplest method for determination of individual ipecac alkaloids. In 4 *N* methanolic ammonia emetine has an absorption maximum between 282 mμ and 286 mμ as determined with the Cary recording spectrophotometer. The value for $E(1\%, 1\text{ cm.})$ at 285 mμ was found to be 161.5. The absorption spectrum for cephaeline in the same solvent is very similar to that of emetine and has a maximum at 282 mμ. Ultraviolet spectrophotometry was used for determining the amounts of emetine and cephaeline in the alcoholic eluates obtained from Dowex 50-X_2. This method was not entirely satisfactory for the acid eluates of cephaeline from the anionic resin. Small amounts of impurities are eluted with the alkaloid giving a high background absorption. The phenolic alkaloids can easily be determined by various colorimetric methods of considerable specificity. They will, for example, couple with diazonium salts in alkaline media to give intensely colored solutions. Diazotized sulfanilamide, sulfanilic acid

TABLE I.—DETERMINATION OF EMETINE AND CEPHAELINE BY ION EXCHANGE

	Emetine			Cephaeline		
Treatment	Added, mg.	Recovered mg.	Recovered %	Added, mg.	Recovered mg.	Recovered %
Dowex 50-$X_2(H^+)$	3.24	3.26	100.7	1.02	1.02	100.0
	2.96	2.96	100.0	2.38	2.40	100.9
	5.16	5.12	99.2	..	..	...
Dowex 1-$X_1(OH^-)$	..	..	...	2.04	2.03	99.5
	..	..	...	3.41	3.42	100.2
Dowex 50-$X_2(H^+)$	6.24	6.20	99.4	2.51	2.49	99.2
Dowex 1-$X_1(OH^-)$	2.75	2.75	100.0	2.76	2.76	100.0

EXPERIMENTAL

Ion Exchange Studies with Pure Alkaloids.—The ion exchange resins, the extraction tubes and the ion exchange columns used in this work have been described earlier (6). When working with pure alkaloid solutions, glass wool was placed in the stopcock of the extraction tube. This served as a coarse filter. In such an extraction tube 25 ml. of an aqueous solution of the alkaloidal salt, corresponding to 1 to 7 mg. of base, was shaken mechanically for 10 minutes with 1 Gm. of Dowex® 50-$X_2(H^+)$. The liquid was drained off and the resin washed with a little distilled water. The alkaloid was eluted from the resin with 4 *N* ammonia in 70% v/v methanol. The eluate was collected in a 100-ml. volumetric flask and analyzed. In other experiments the eluate was allowed to flow through a 10 to 15 cm. long column of Dowex 1-X_1 (OH^-) before it was collected and analyzed for emetine. The anionic resin was then washed with water until it was free of ammonia, and cephaeline was eluted with 0.1 *N* acetic acid. This eluate was also collected in a 100-ml. volumetric flask and analyzed. In a third type of experiment the aqueous solution of or *p*-nitroaniline may be used. We chose diazotized *p*-aminoacetophenone as our coupling agent and used the reagents described by Hochberg, *et al.* (8) for colorimetric determination of thiamine. An orange-red color is produced almost immediately and is reasonably stable. It has an absorption maximum at 495 mμ.

Assay of Ipecac

In general, ipecac is extracted more easily than cinchona and nux vomica (9). This was also found to be the case when the extraction was carried out by ion exchange resins. A sample of about 200 mg. of ipecac gave the proper amounts of alkaloids in the eluates. Such a sample was completely extracted by 1 Gm. of Dowex 50-X_2 in 15 minutes at room temperature. The yield of alkaloids could not be increased further by increasing the temperature, the time, or by addition of formic acid as described in our previous paper (6). On the basis of a number of experiments carried out under different conditions, we have arrived at the following as the simplest possible method.

Extraction.—Place 0.2 Gm. of finely powdered ipecac, accurately weighed, in an extraction tube. Add 1 Gm. of activated Dowex 50-X_2 and 25 ml. of

[1] Cephaeline hydrochloride was obtained through the courtesy of The Wellcome Research Institution, London.

distilled water. Shake mechanically for 15 minutes. Transfer the mixture quantitatively to a suitable ion exchange tube and remove the crude drug residue by means of a backwash with distilled water (6).

Purification and Determination of Nonphenolic Alkaloids.—Allow the water to drain off and elute the alkaloids with 4 *N* ammonia in 70% v/v methanol. Let the eluate flow directly through a column of Dowex 1–X_1 previously activated with 1 *N* sodium hydroxide. A column which is 10 to 15 cm. in height is satisfactory. Collect 100 ml. in a volumetric flask. Determine the alkaloids by ultraviolet spectrophotometry at 285 mμ, using a blank which has been obtained by passing methanolic ammonia through the same column of Dowex 1–X_1. Calculate the result as emetine.

Determination of Phenolic Alkaloids.—Wash the anionic exchange column with enough distilled water to remove the ammonia. Elute the phenolic alkaloids with 0.1 *N* acetic acid and collect 100 ml. Pipet 10 ml. of the eluate into a 25-ml. volumetric flask. Add 5 ml. of sodium hydroxide-sodium bicarbonate solution and, after mixing, 5 ml. of the coupling reagent (8). Adjust the volume to the mark with distilled water. Determine the color intensity at 495 mμ after 10 to 15 minutes. Use a blank prepared from the same alkaloidal eluate without the coupling reagent. Calculate the per cent of phenolic alkaloids as cephaeline.

The results obtained by this method as well as by the official assay procedure are given in Table II. When the alkaloid solution obtained in the official assay had been titrated, it was made strongly basic with sodium hydroxide, and the nonphenolic fraction was extracted with ether. The result was calculated as emetine. The phenolic alkaloids were determined by difference and calculated as cephaeline. In separate experiments the extraction procedure of the U. S. P. was combined with the ion exchange method for purification and separation of the alkaloids.

TABLE II.—ASSAY OF IPECAC[a]

Method	Nonphenols, %	Phenols, %	Total Alkaloids, %
U. S. P. XV	1.33 ± 0.02	0.90 ± 0.01	2.26 ± 0.02
U. S. P. Extraction with Ion Exchange Purification	1.33 ± 0.01	0.91 ± 0.01	2.24 ± 0.01
Ion Exchange Extraction and Purification	1.41 ± 0.02	1.02 ± 0.01	2.43 ± 0.01

[a] The results are average values of at least three determinations.

DISCUSSION

When aqueous solutions of salts of emetine and cephaeline were shaken with the cationic exchange resin, the alkaloids were quantitatively removed from the solutions. They could be recovered without loss by elution with alcoholic ammonia (Table I). Emetine was not affected by being passed through the anionic exchange resin, whereas cephaeline was adsorbed. In our earlier work with opium (7), we had observed that it was not necessary to convert the anionic resin completely to the hydroxide form with sodium hydroxide. Washing the resin with alcoholic ammonia would activate it sufficiently for adsorption of morphine. Under these conditions cephaeline did not give consistent results. The recoveries varied from 93 to 100 per cent. Activation with 1 *N* sodium hydroxide invariably led to a 100 per cent recovery upon elution with acid. The reason for this difference is possibly that cephaeline, with its two basic nitrogens, is a weaker acid than morphine.

When the crude drug was assayed by the ion exchange method, the anionic resin effectively removed the colored impurities from the ammoniacal eluate. Only a small amount of these impurities was later eluted with acetic acid.

Table II shows that the official assay gave lower results than the ion exchange procedure. When the official extraction method was combined with an ion exchange purification, the results were the same as those obtained by the U. S. P. method. It must, therefore, be concluded that the method of extraction used in the official assay is inefficient and is responsible for the low results. This inefficiency was most pronounced for the phenolic alkaloids. Extraction of these was 10.8 per cent too low as compared with 5.7 per cent for the nonphenolic alkaloids. This must be due to the fact that the nonphenolic alkaloids of ipecac are much more soluble in ether than are the phenolic alkaloids.

Emetine, dissolved in methanolic ammonia, does not absorb ultraviolet light at 310 mμ. The nonphenolic fraction of the ipecac alkaloids showed an absorbance of 0.02 at this wavelength. It seems safe to assume that this absorption is due to the presence of methylpsychotrine and emetamine, both of which have a high optical density at 310 mμ.

The equivalent weight used for the ipecac alkaloids in the official assay is too large. It corresponds to a mixture of 95 per cent emetine and 5 per cent cephaeline. Rio ipecac usually contains about 75 per cent of nonphenolic alkaloids. In the Cartagena variety this fraction accounts for only 40 to 60 per cent (1). It follows, therefore, that the titration result obtained in the U. S. P. method will indicate a slightly higher content of alkaloids than is actually present in the titrated solution. This is illustrated in Table II, which shows that the sum of the nonphenolic and the phenolic fractions (2.23%) is less than the per cent of total alkaloid (2.26%).

SUMMARY

The ion exchange method for extraction of alkaloids from crude drugs—described earlier for cinchona, nux vomica, and opium—has been shown to give equally satisfactory results with ipecac. An extraction time of fifteen minutes at room temperature is sufficient for complete extraction. By means of an anionic exchange resin the extracted alkaloids are purified and separated into phenolic and nonphenolic fractions. The alkaloids are determined by spectrophotometric procedures.

This method gives results which are appreciably higher than those obtained in the official

assay. It is simple, rapid and has a high degree of precision.

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Notes

A Note on The Effect of Vitamin B_{12} on the Unsaturated Fatty Acid Content of Castor Oil*

By NOURI Y. MARY, RICHARD H. BOHNING, and JACK L. BEAL

IT IS WELL KNOWN that several vitamins have been proved to be essential to plant growth. This is particularly true of the B vitamins. Very little investigation, however, has been carried out to determine whether or not vitamin B_{12} has a role in plant metabolism. Experimental work (1, 2) has shown that vitamin B_{12} plays an important role in carbohydrate metabolism in animals. It has also been suggested that vitamin B_{12} may play a part in the conversion of linoleic and linolenic acids into more highly unsaturated fatty acids (3).

Since castor oil contains (4) approximately 5.4% linoleic acid and 92.6% ricinoleic acid, *Ricinus communis* L. var. Baker 195 was chosen as the test plant for investigating whether or not vitamin B_{12} has an effect on the amount of its unsaturated fatty acids.

The plants were started from seed[1] and maintained by sand culture. The culture solution contained per liter the following milliliters of 1 *M* solutions of various salts: 2 of potassium nitrate, 2 of potassium dihydrogen phosphate, 3 of calcium nitrate, and 2 of magnesium sulfate. In addition, 1 ml. of a micrometabolic element solution was added to each liter of culture solution. The vitamin B_{12}[2] was added directly to the nutrient solution of all test plants. All plants were harvested at the age of six months. The seeds were ground in a glass mortar and the ground material extracted with ether in a Soxhlet extractor. The ether solution was filtered and the ether then removed from the samples by vacuum distillation. The iodine value was determined for each sample by means of the U. S. P. XIV method (5). This value served as an indication as to the amount of unsaturated fatty acids in each sample. The results are listed in Table I.

As can be observed in Table I, vitamin B_{12} had no significant effect on the amount of unsaturated fatty acids present in the samples. There was no noticeable effect of vitamin B_{12} on the growth of the plants nor was there a significant difference in the yield of seed from treated and nontreated plants.

TABLE I.—IODINE VALUES OF THE CASTOR OIL SAMPLES

Amount of B_{12}, mg./L.	No. of Plants Represented	Iodine Values[a]
Control No. 1	2	84.5
Control No. 2	2	85.5
Control No. 3	1	84.8
0.5 mg. B_{12}	3	85.5
1.0 mg. B_{12}	3	85.1
3.0 mg. B_{12}	3	84.0
5.0 mg. B_{12}	1	84.0
5.0 mg. B_{12}	1	84.0
5.0 mg. B_{12}	1	84.8
10.0 mg. B_{12}	3	85.0
Control No. 1; no iron	2	83.5
Control No. 2; no iron	2	84.9
1.0 mg. B_{12}; no iron	2	83.9
5.0 mg. B_{12}; no iron	2	84.3
10.0 mg. B_{12}; no iron	2	83.9

[a] Average of two determinations.

* Received October 31, 1955, from College of Pharmacy and Department of Botany and Plant Pathology, The Ohio State University, Columbus 10.

[1] We are indebted to The Baker Castor Oil Company, Bayonne, N. J., for the supply of seeds.

[2] We are indebted to Merck and Company, Inc., Rahway, N. J., for the supply of vitamin B_{12}.

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The Antituberculous Activity of some Isonicotinic Acid Hydrazide Congeners and Derivatives*

By FLOYD E. ANDERSON, GODFREY F. GRAIL, FREDERICK LEONARD, LEON E. TENENBAUM, JOHN J. D. HART, and FRED A. BARKLEY

Antimycobacterial data for a number of previously unreported congeners and derivatives of isonicotinic acid hydrazide are presented.

FOLLOWING THE almost simultaneous discovery of the antituberculous activity of isonicotinic acid hydrazide (INH) in the laboratories of Bayer (1), Hoffmann-La Roche (2), and Squibb (3), a considerable number of cognate compounds were prepared and examined to explore the structure-activity relationships (4–19) of this clinically important agent.

In continuation of our search (20–22) for effective antitubercular substances we systematically screened a variety of hydrazines derived from aromatic and heterocyclic carboxylic acids. Subsequent to our preparation of these compounds, several articles were published in which were described compounds identical to those synthesized in these laboratories. In this communication we wish to report the synthesis of a number of compounds hitherto not described and their *in vitro* activity against two strains of pathogenic mycobacteria. Activity data for several compounds previously reported are included for purposes of comparison.

Antimycobacterial activity data in Table I confirm the decreased effectiveness (4, 6, 8, 9, 10) which usually results from the presence of additional substituents on the pyridine ring of INH. Vejdlek (12) reported "strong" tuberculostatic effects *in vitro* for 2-aminoisonicotinic acid hydrazide while Beyerman and Bontekoe (9) stated that 2,6-diaminoisonicotinic acid hydrazide was without activity. We observed promising activity in a related compound, 2-(*n*-butylamino)isonicotinic acid hydrazide (No. 2112); *in vivo* experiments with this compound continue.

Activity is reduced to an unpromising level when the second hydrazine nitrogen of INH is substituted with the alpha-pyridine moiety (No. 1581). This is in agreement with and an extension of the findings of Fox and Gibas (5) who noted that the isomeric 1-isonicotinoyl-2(4-pyridyl) hydrazine was inactive *in vivo*. It is interesting to note that the sulfonic acid analog (No. 1551) of nicotinic acid hydrazide (No. 1507) is completely without activity.

The compounds presented in Table II represent in part the effort made to prepare derivatives of the INH molecule to obtain certain chemotherapeutic advantages. For example, in some gastrointestinal forms of tuberculosis advantage might result from a less soluble form of the drug. Sterilization of the gut by sparingly soluble sulfa drugs (23, 24) is successfully practiced.

Other objectives sought through INH derivatives were: a delayed detoxification and elimination of the drug, local tissue concentration effects, repository forms to effect protracted and constant serum concentrations, diminished neurotoxicity (25), a decreased rate of resistance development by the tubercle bacillus and synergistic effects between INH and INH congeners (21, 22, 27). The evaluation of

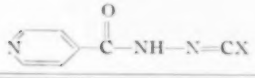
TABLE I

Compound No.	Hydrazine[a]	Empirical Formula	m.p., °C.	—Anal.—Nitrogen— % Calcd.	% Found	Minimal Bacteriostatic Concentration[b] mg. % H37RV	BI
1507	Nicotinoyl[c]	$C_6H_7N_3O$	158–159	...	...	4.0	16.0
1512	6-Hydroxy-nicotinoyl[d]	$C_6H_7N_3O_2$	310 dec.	27.5	27.5	64.0	32.0
1551	3-Pyridine-sulfonyl	$C_5H_7N_3O_2S$	95–97	24.3	23.9	>64.0	>64.0
1497	Isonicotinoyl[c]	$C_6H_7N_3O$	167–170	...	...	0.007	0.007
1506	2,6-Dihydroxy-isonicotinoyl[e]	$C_6H_7N_3O_3$	230	24.8	24.6	16.0	32.0
3112	2-(*n*-Butylamino) isonicotinoyl	$C_{10}H_{16}N_4O$	134–136	26.9	26.8[f]	0.05	...
1581	1-(Isonicotinoyl)-2-(alpha-pyridyl)	$C_{11}H_{10}N_4O$	163	26.2	26.1	16.0	16.0
1874	1-(2-Pyrimidyl)-2-(*o*-hydroxy-benzylidene)	$C_{11}H_{10}N_4O$	213–215	26.2	25.9	0.125	...

[a] All compounds are named as derivatives of hydrazine rather than acid hydrazides to permit inclusion of numbers 1581 and 1874.
[b] For technique see (21). Mg. % is the number of mg. of compound per 100 ml. nutrient medium which completely inhibits the growth of the mycobacteria.
[c] Activity reference compound, see (1–3). Compound 1497 is Isoniazid.
[d] Mills, W. H., and Widdows, S. T., *J. Chem. Soc.*, **93**, 1381(1908) reported a m.p. 310°.
[e] Beyerman, H. C., and Bontekoe, J. S., (9), reported a m. p. 235° and indicated it had no antituberculous activity. Isler, O., *et al.* (10), report its lack of *in vivo* activity.
[f] Calcd.: C, 57.67; H, 7.75. Found: C, 57.54; H, 7.79.

* Received October 17, 1955, from the Research Laboratories of Nepera Chemical Company, Inc., Yonkers, N. Y.

TABLE II



Compound No.	Carbonyl Component (=CX)	Empirical Formula	m. p., °C.	Anal.—Nitrogen— % Calcd.	% Found	Minimal Bacteriostatic Concentration[a] mg. % H37RV	BI
1903	2,6-Dihydroxybenzaldehyde	$C_{13}H_{11}N_3O_3$	247–248	16.3	15.9	0.015	...
1712	Ethyl phenylglyoxylate	$C_{16}H_{15}N_3O_3$	104–106	14.1	14.2	32.0	>64.0
2000	2,2,5,5-Tetramethyltetrahydro-3-furanone	$C_{14}H_{19}N_3O_2$	205–207	16.0	15.7	0.031	...
1711	2,2,6,6-Tetramethyl-4-piperidone	$C_{15}H_{22}N_4O$	200–201	...	...	64.0	>64.0
1738	Benzophenone	$C_{19}H_{18}N_2O$	130.5–131.5	14.0	14.1	2.0	0.5
1549	Glucuronolactone[b]	$C_{18}H_{20}N_6O_7$	229–230	19.5	19.3	2.0[c]	0.062

[a] For technique see (21).
[b] Analysis corresponds to equivalent of two moles INH and one mole glucuronolactone (28).
[c] Partial inhibition to 0.007 mg. % concentration.

some compounds in Table II will be reported in later publications. Compound No. 1549 is currently being tested clinically. This compound (28) is the interaction product of one mole of glucuronolactone and two moles of INH in the solvent pyridine; it differs from the similar composition reported by Sah (26) which contains the equivalent of one mole each of INH and glucuronolactone. The synthesis of all compounds followed well-established general procedures (6, 11).

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A Note on the Antiseptic Value of Certain Pectin Pastes*

By JACK N. BONE and CHARLES F. POE

SINCE the seventh National Formulary first recognized pectin as an official compound, very little research has been published on the effect of common antiseptics and dermatological medicinals incorporated in pectin pastes.

The object of the present communication is to determine the antiseptic effects of certain common dermatological medicinals in pectin pastes. Comparisons also are made as to the antiseptic value of phenol incorporated in the U. S. P. phenol ointment and in pectin paste. The stability of pectin pastes containing different medicinal agents also is presented.

There are two formulas for pectin pastes given in the N. F. IX. One contains 7.5% pectin and the other contains 3.5% pectin. The heavier paste was used in the present experiments.

The agar cup-plate method recommended by Food and Drug Administration (1) was used to test the antiseptic power of the pastes. Ten per cent horse serum was added to the agar because pectin pastes are frequently used on open wounds, cuts, or in the presence of serous fluid. The test organism was *Micrococcus pyogenes* var. *aureus*.

Table I includes the experimental data showing the effects of the different medicinal agents in the pectin pastes. In order to determine whether or not the material under test was germicidal or just inhibitory, a small amount of the agar in the clear zone was subcultured into sterile nutrient broth and incubated 48 hours at 37°. If the broth tubes showed no growth, it was known that the paste had germicidal activity; if growth appeared, the paste exhibited only inhibitory action. All tests were run in triplicate and the widths of the zones in the tables are an average of the three determinations.

In order to compare the antiseptic value of phenol when incorporated in a pectin paste with that incorporated in the U. S. P. phenol ointment base, various samples were prepared. The different samples of pectin paste and U. S. P. phenol ointment contained 2, 4, 6, 8, and 10% phenol. Each sample was tested with the serum agar cup-plate method, and the inhibitory and germicidal actions were recorded. Phenol, when incorporated in pectin paste, is slightly inhibitory in concentrations of 2 and 4%. In the U. S. P. ointment base, it has no activity in these amounts. Phenol, in amounts from 6 to 10% in both bases, showed about the same penetrating power, but in pectin paste the action is bactericidal and in the U. S. P. ointment the action is bacteriostatic. Prout and Strickland (2), Bryan (3), Reddish and Wales (4), and Clark (5) have reported that phenol in varying amounts up to 10% in the U. S. P. ointment base had little or no antiseptic value.

Little or no antiseptic effect in the amounts used was shown by the pectin pastes containing phenol in amounts less than 6%, by cresol, 1%, coal tar 5%, thymol iodide 5%, benzoic acid 0.2%, sulfanilamide 5%, sulfur 5%, picric acid 1%, tannic acid 10%, acriflavine 1%, iodoform 4%, and oil of birch tar 10%.

Most of the pectin pastes were stable for three months when stored at room temperature except under the following conditions: (*a*) The *p*H range of the finished product was above 6.5. (*b*) The presence of iodine caused hardening of the paste. (*c*) The medicinals used were insoluble in the *p*H range of the paste, i.e. about 4.5.

TABLE I.—INHIBITION ZONES CAUSED BY MEDICINAL AGENTS IN PECTIN PASTES

Added Ingredient, %	Width, mm.	Action[a]
Benzoic, 6; salicylic, 3	6	−
Salicylic, 3	5	+
Salicylic, 3; sulfur, 5	5	−
Tannic acid, 20; acriflavine, 0.1; Na_2SO_3, 0.2	5	−
Tannic acid, 2.5; $HgCl_2$, 0.1; Na_2SO_3, 0.2	5	−
Boric acid, 5	5	+
Boric acid, 10	6	+
Boric acid, 5; phenol, 1	5	+
Sodium sulfathiazole, 5	2	+
Gentian violet, 1	7	−
Brilliant green, 1	4	−
Brilliant green, 1; acriflavine, 1	5	−
Iodine, 2; KI, 2.5	12	−
Resorcinol, 10	5	−
Phenol, 6	3	−
Phenol, 8	6	−
Phenol, 10	8	−

[a] + inhibitory; − germicidal.

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* Received June 9, 1955, from the College of Pharmacy, University of Colorado, Boulder.

A Note on the Solubility of Strychnine in Alcohol and Water Mixtures*

By WALLACE F. NG and CHARLES F. POE

THE JUNIOR AUTHOR was employed by the State of Colorado as an expert witness in a murder trial in which the defendant was accused of adding strychnine to a bottle of whiskey; this action resulted in the death of a man who drank one small glass of the whiskey. The question arose whether or not the amount of strychnine recovered from the body would be soluble in one ounce of fifty-proof whiskey.

A search of the literature on this subject revealed that a very small amount of data was available on the solubility of strychnine in ethyl alcohol, especially in mixtures containing from 40 to 60% of alcohol. Therefore, the authors decided to determine the solubility of strychnine base and strychnine sulfate in mixtures of ethyl alcohol and water, and in mixtures of isopropyl alcohol and water in concentrations from 0 to 95% by volume and at temperatures from 20 to 75°. The results for the solubility of the strychnine base are reported in this communication.

Seidell (1) lists the solubility of strychnine at 20° in alcohol-water mixtures containing from 20 to 90% ethyl alcohol. The solubilities varied from 0.024 to 0.59 Gm. per 100 ml. of solvent. Lange (2) states that 0.91 Gm. of strychnine is dissolved in 100 ml. of cold 95% ethyl alcohol.

MATERIALS AND PROCEDURE

Reagent grade chemicals were selected. Aqueous solutions of the alcohols were prepared by diluting ethyl alcohol and isopropyl alcohol with distilled water after each alcohol had been redistilled several times. The concentrations were determined accurately from their densities. Each solution was checked for refractive index with the Abbé refractometer.

The sample of strychnine was reduced to a fine powder before use to increase the rate of solution. In each determination, an excess of powdered strychnine base was added to 50 ml. of aqueous alcoholic solution contained in a large Pyrex tube, the tube then was stoppered. The test tube was placed in a beaker of water and heated for one-half hour with frequent agitation to approximately ten degrees above the desired temperature. The test tube then was placed in a constant temperature bath, capable of being regulated to 0.05°, and allowed to remain in the bath several hours at a given temperature with frequent agitation in order to prevent supersaturation and to permit an equilibrium to be reached between the solute and solvent.

The excess strychnine was permitted to settle, and three samples of the supernatant saturated solution were withdrawn by means of a dry 5-ml. volumetric pipet. Previous to withdrawal of the sample, a piece of glass tubing about 6 cm. in length was attached to the lower orifice of the pipet. A pledget of cotton was placed in this piece of glass tubing to serve as a filter. In each determination, the pipet first was heated in a dry air oven to the same temperature as that of the solution to be withdrawn. This procedure prevented the strychnine from crystallizing out in the pipet. Speed of operation was essential. The samples of the saturated solution were transferred to tared dishes. The solvent was allowed to evaporate spontaneously on a steam bath, care being taken near the end of the process to avoid spattering. The residue then was dried to constant weight in an air oven at 100° and cooled in a desiccator before weighing. Calculations were made on the basis of grams of strychnine dissolved in 100 ml. of solution.

EXPERIMENTAL RESULTS

The results of the solubility tests are shown in a series of tables and figures. Table I gives the grams of strychnine soluble in various percentages of ethyl alcohol at different temperatures. Table II gives similar information when isopropyl alcohol was used as the solvent. Figures 1 and 2 show the curves obtained with constant percentages of ethyl and isopropyl alcohols when solubilities were plotted against temperatures. Figures 3 and 4 give the isothermal curves obtained when solubilities were plotted against various percentages of the alcohols.

From an examination of the tables and curves, it is evident that the solubility of strychnine increases

TABLE I.—GRAMS OF STRYCHNINE SOLUBLE IN 100 ML. OF SOLUTION OF DIFFERENT ETHYL ALCOHOL–WATER MIXTURES

% by Vol.	20°	30°	40°	50°	60°	75°
95	0.52	0.70	0.92	1.10	1.32	1.58
90	0.70	0.99	1.16	1.41	1.62	1.80
80	0.83	1.09	1.34	1.52	1.73	1.91
70	0.65	0.94	1.18	1.38	1.59	1.81
50	0.29	0.39	0.50	0.66	0.85	1.12
30	0.06	0.09	0.13	0.17	0.25	0.34
0	0.014	0.020	0.034	0.040	0.045	0.060

TABLE II.—GRAMS OF STRYCHNINE SOLUBLE IN 100 ML. OF SOLUTION OF DIFFERENT ISOPROPYL ALCOHOL–WATER MIXTURES

% by Vol.	20°	30°	40°	50°	60°	75°
95	0.42	0.62	0.82	1.04	1.30	1.69
90	0.67	0.88	1.13	1.36	1.64	2.26
80	0.81	1.06	1.32	1.64	2.13	2.86
70	0.70	0.98	1.20	1.54	1.91	2.60
50	0.37	0.53	0.69	0.87	1.03	1.29
30	0.13	0.14	0.16	0.23	0.29	0.46
0	0.014	0.020	0.034	0.040	0.045	0.060

* Received June 9, 1955, from the College of Pharmacy, University of Colorado, Boulder.
Presented to the Pharmacy Section, A.A.A.S., Berkeley, Calif., December 1954.

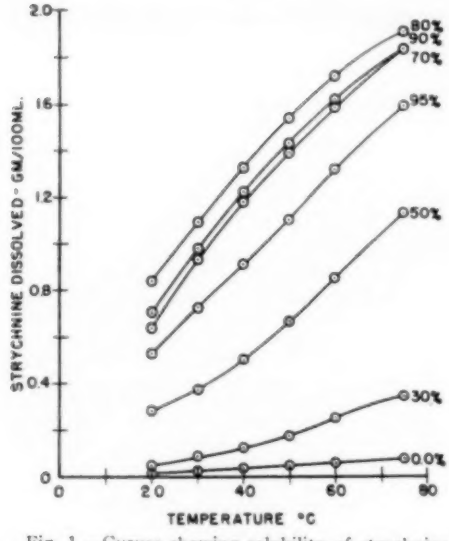

Fig. 1.—Curves showing solubility of strychnine in ethyl alcohol-water mixtures at different temperatures.

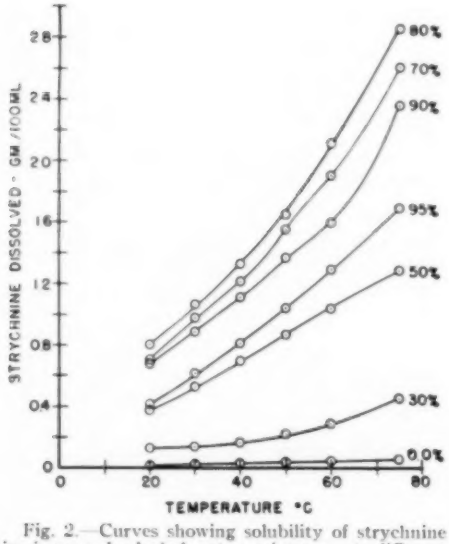

Fig. 2.—Curves showing solubility of strychnine in isopropyl alcohol-water mixtures at different temperatures.

with an increase in the concentration of the alcohols up to a maximum in the 80% mixture for both ethyl and isopropyl alcohols. From this point, the solubility decreases as the percentages of both alcohols increase and as the temperatures increase.

The solubility of strychnine in isopropyl alcohol was about the same as in ethyl alcohol from 0 to 95% and up to 60°. However, at 60 and 75° isopropyl alcohol in strengths of 70 and 80% showed somewhat larger amounts of strychnine dissolved.

Several investigators have found solubilities to reach a maximum and then decrease when compounds are dissolved in a mixture of two solvents, one of which is organic. When working with

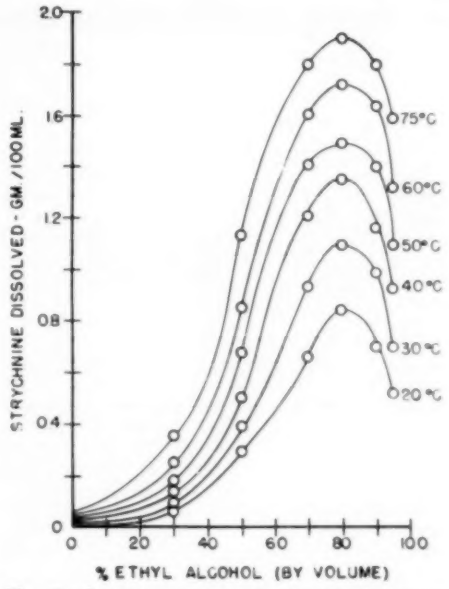

Fig. 3.—Isotherm curves of ethyl alcohol-water mixtures showing solubility of strychnine.

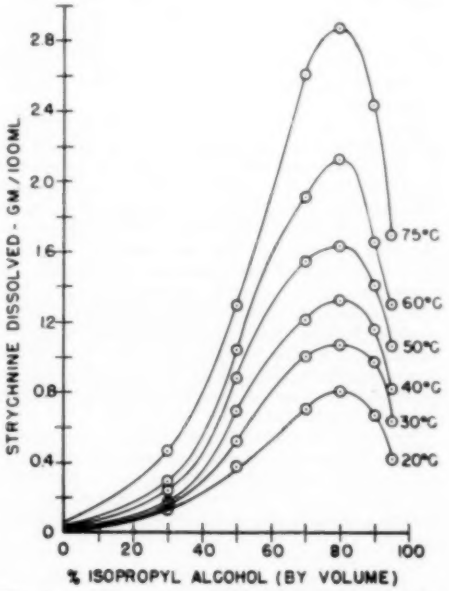

Fig. 4.—Isotherm curves of isopropyl alcohol-water mixtures showing solubility of strychnine.

organic compounds, Herz and Knoch (3) found that this condition takes place when mixtures of acetone and water were used as solvents. Seidell (4), Dehn (5), Gregg-Wilson and Wright (6), and Holleman and Antusch (7) noted similar phenomena with alcohol-water solvents. Various explanations have been offered for this condition including the following: (*a*) The solute forms a molecular compound; (*b*) the solute combines with one of the solvents; (*c*) the solute forms a molecular compound with both solvents; (*d*) with complex organic compounds each solvent acts differently on various groups in the complex compound.

CONCLUSIONS

1. The solubilities of strychnine in ethyl- and isopropyl-water mixtures have been determined from 20 to 75°.

2. The solubilities of strychnine in both ethyl- and isopropyl-water mixtures reach a maximum in the 80% mixtures.

3. The solubilities increased in all mixtures as the temperature increased.

4. The solubilities of strychnine in mixtures of the two alcohols were nearly the same except that isopropyl alcohol mixtures of 70 and 80% show greater solvent action at 60 and 75°.

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A Note on the Preparation of 4-Chloro-2-isopropyl-5-methylphenoxyacetamidine Hydrochloride*

By DONALD F. WALKER†

4-Chloro-2-isopropyl-5-methylphenoxyacetamidine hydrochloride was prepared in this laboratory for evaluation in systemic fungous infections. The compound was prepared by condensing (1) 4-chloro-2-isopropyl-5-methylphenol with chloroacetonitrile to form the aryloxy acetonitrile which was reacted with ethanolic hydrochloric acid to give ethyl 4-chloro-2-isopropyl-5-methylphenoxyacetimidate hydrochloride. This imido-ester hydrochloride was reacted with alcoholic ammonia to form the desired amidine (2, 3).

4-Chloro-2-isopropyl-5-methylphenoxyacetamidine hydrochloride was active against *Histoplasma capsulatum* and other representative pathogenic fungi in the broth dilution test.[1] Detailed results of the antifungal testing of these compounds will be reported elsewhere.

EXPERIMENTAL

4-Chloro-2-isopropyl-5-methylphenoxyacetonitrile.—The condensation of chloroacetonitrile with 4-chloro-2-isopropyl-5-methylphenol in methyl ethyl ketone resulted in a 65% yield of a low melting solid. Recrystallization from low boiling petroleum ether gave the product, m. p. 55–57° (uncorr.).

Anal.—Calcd. for $C_{12}H_{14}ClNO$: C, 64.43; H, 6.30. Found: C, 64.18; H, 6.55.[2]

Ethyl 4-chloro-2-isopropyl-5-methylphenoxyacetimidate hydrochloride.—The preceding compound was dissolved in chloroform and treated with ethanol and anhydrous hydrogen chloride in the cold. The imido-ester hydrochloride was precipitated by the addition of anhydrous ether. The yield was 82%, m. p. 113–114° (uncorr.). This crude material was not recrystallized in order to avoid formation of the amide on warming (4).

Anal.—Calcd. for $C_{14}H_{21}ClNO_2.HCl$: C, 54.90; H, 6.91; Neutral Equiv., 306.2. Found: C, 55.66; H, 7.26; Neutral Equiv., 297.8.

4-Chloro-2-isopropyl-5-methylphenoxyacetamidine hydrochloride.—The imido-ester hydrochloride and alcoholic ammonia gave the desired amidine hydrochloride after standing with intermittent shaking for forty-eight hours. Recrystallization from a mixture of ethanol and methyl ethyl ketone resulted in a 36% yield, m. p. 170–171° (uncorr.).

Anal.—Calcd. for $C_{12}H_{17}ClN_2O.HCl$: C, 51.99; H, 6.55. Found: C, 52.14; H, 6.73.

SUMMARY

Three new compounds are reported. 4-Chloro-2-isopropyl-5-methylphenoxyacetamidine hydrochloride was active against *Histoplasma capsulatum* and other pathogenic fungi.

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* Received Dec. 14, 1955, from the Research Laboratories, Parke, Davis & Company, Detroit, Mich.
† Present address: *Chemical Abstracts*, Ohio State University, Columbus 10, Ohio.
[1] Antifungal screening was performed in this laboratory by Dr. A. B. Hillegas and associates.
[2] Microanalyses were performed by C. E. Childs and associates in our laboratory.

A Note on Tablet Hardness*

By EINO NELSON†

IN A RECENT PUBLICATION experimental evidence was presented to show that pressure distribution within the die cavity was not uniform during tablet compression (1). The results obtained suggested that a difference in intergranule bonding strength might be encountered between sections of a tablet parallel and normal to the direction of compression. It was recognized that if distortion of granule shape resulted from compression it might be difficult to confirm this observation by hardness tests. This note describes a method for making cubical tablets and presents the results from testing their hardness in sections parallel and normal to the direction of compression when such tablets were made from a given granule size over a range of compression pressures and when made from various sized granules at constant pressure.

EXPERIMENTAL

Cubical tablets were made by carrying out compressions in a $^1/_4$ inch square die and adjusting fill and pressure by trial and error until the tablets resulting were $^1/_4$ inch high. The tablets were made from a sulfathiazole granulation formed by adding 5% starch paste to sulfathiazole powder, granulating and drying. Six series of ten tablets each were made from 20/30 mesh granules in a pressure range from 250 to 1350 Kg. per sq. cm. on the lower punch. Another series of tablets was made at a lower punch pressure of 750 ± 25 Kg./sq. cm.; ten each from 20/30, 30/40, 40/60, 60/80, and 80/100 mesh granules. Almost all tablets used in the tests were within 0.005 inch of being perfect cubes. The machine used to make the compressions has been previously described (2).

The measure of hardness was the force on a knife edge in contact with the face of a tablet needed to cause it to cleave. The apparatus used to determine this force consisted of a piece of square brass stock drilled at one end for fastening to a support and split a short distance from this pivot end to allow insertion of a piece of knife blade. A small bucket was suspended at the other end of this bar. In making tests the bar with the edge of the knife blade in contact with the cubical tablet was loaded by pouring sand into the bucket until the tablet cleaved. The bucket and its contents were weighed and this weight multiplied by the lever arm ratio plus the weight due to the loading caused by the bar itself was taken to be the force necessary to cause cleavage.

Of ten tablets made at each pressure or from each granule size, five were tested with the knife edge placed to cause the tablet to cleave parallel to the direction the tablet was compressed and five with the knife edge placed so the tablet would cleave in a plane normal to the direction of compression. The median value of force in each of the five trials was taken to represent hardness.

The relation between hardness and compression pressure found in testing the tablets is shown in Fig. 1. It was expected from the measurements previously cited (1) that the position of the curves

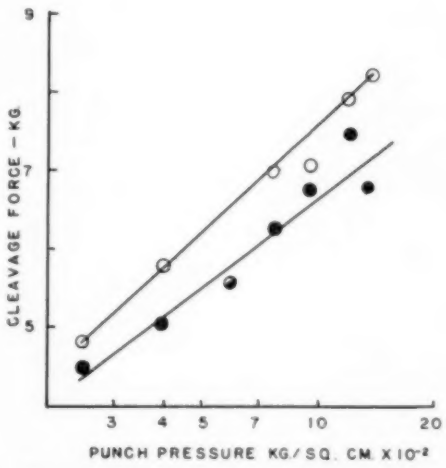

Fig. 1.—Force necessary to cause cleavage of cubical tablets as a function of compression pressure; ○-section of tablet parallel to direction of compression; ●-section of tablet normal to direction of compression.

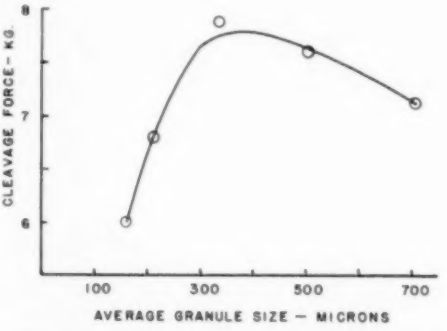

Fig. 2.—Force necessary to cause cleavage of cubical tablets in sections parallel to direction of compression as a function of granule size.

would be reversed since less pressure is found on the die wall than on the punches. The fact that tablets were harder in planes parallel to the direction of compression is probably due to distortion of the granules. There is now evidence available to indicate that granules tend to "pancake" during compression (3). In testing hardness then, with the blade parallel to the direction of compression, there were more granules to cut per unit area than bonds to part between granules than there were when the blade was placed normal to the direction of compression. This plot also shows that there may be a tendency for the difference in hardness between

* Received July 18, 1955, from the School of Pharmacy, University of Wisconsin, Madison 6.
† Present address: University of California, School of Pharmacy, San Francisco, Calif.

parallel and normal sections to diminish at low pressures.

The relation between tablet hardness in a plane parallel to the direction of compression and average particle size for tablets made at constant pressure is shown in Fig. 2. The difference in hardness between parallel and normal section when plotted against the same abscissa was qualitatively similar to this curve. This plot is not shown. In Fig. 2 the abscissa, average particle size, is the arithmetic mean of the sieve openings in microns between which the granules were collected.

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Book Notices

Reagent Chemicals and Standards. 3rd ed. By JOSEPH ROSIN. D. van Nostrand Company, Inc., New York, 1955. x + 561 pp. 16 x 23.5 cm. Price $9.50.

This useful book, now in its third edition, provides basic information on the properties, standards, and tests for more than six hundred reagent chemicals. In the course of the revision nearly fifty new reagents and solvents have been added. Among the new reagents are: anthrone, cyclohexane, dimethylformamide, ethylene-diamine-tetraacetic acid and its disodium salt, eriochrome black T, isooctane, rhodamine B, tetramethylammonium hydroxide, triphenyltetrazolium chloride and vanadyl sulfate.

In general, the style employed for the monographs is essentially the same as that of the second edition which was published in 1946. Limit tests for trace impurities and assays are provided where they are considered to be essential and physical characteristics such as optical rotation, melting point, and specific gravity, are included in monographs where applicable and significant.

New features include directions for preparing reagent solutions, directions for carrying out the common physical and chemical determinations, graphical formulas for most of the organic compounds, and tables of useful analytical data such as buffer mixtures and indicator solutions.

Dr. Rosin's *Reagent Chemicals and Standards* is more comprehensive than any other comparable treatise in the Engish language.

Methods for Determining Lead in Air and in Biological Materials. Published by American Public Health Association, Inc., New York, 1955. 69 pp. 15 x 22.5 cm. (Paper Cover). Price $1.25.

The text material in this booklet is divided under the headings: collection of samples, preparation of samples for analysis, recommended procedures, dithizone methods, physical methods, and a selected bibliography arranged according to utilization of dithizone, spectrographic, or polarographic methods. The Gambach lead limit test with dithizone as applied to pharmaceutical chemicals and other materials is included.

The American Drug Index. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Company, Philadelphia, 1956. 576 pp. 14.5 x 21 cm. Price $5.

This volume is intended to help those who need information on the nomenclature of pharmaceuticals and chemicals available to the medical and allied professions. In this rapidly expanding field of compounds and products, almost any *new* book is preferable to an old one because it can include the later additions. This book is particularly good because of its extensive coverage of old and newitems. The index is arranged alphabetically with extensive cross-indexing. Names listed are generic, brand (trade mark, proprietary, or specialty), chemical, U. S. P., N. F., N. N. R., and A. D. R.; the official designations referring to U. S. P. XV and N. F. X. Data given in concise form include manufacturer, dosage forms, size, dosage and use. Correlation of individual drugs and combinations occurs throughout the index and large groups of related products are tabulated; e.g., aspirin-barbiturate combinations, belladonna extract combinations, etc.

As in any first edition of a book dealing in many names, some misspellings occur; some source references are incorrect, probably because decisions regarding inclusion in other books such as the U. S. P. XV and N. F. X can be altered after preliminary lists have been distributed; and items mentioned in textbooks but no longer available under the given names are included. Most of these errors are practically unavoidable and do not detract from the value of the index as a reference that should be

useful to physicians, pharmacists, and others who frequently find it necessary to obtain information quickly. The book is a must for pharmaceutical and other reference libraries.

The Complete Book of First Aid. By John Henderson. Duell, Sloan and Pearce, New York—Little, Brown and Company, Boston, 1955. 341 pp. 13 x 22 cm. Price $3.50.

This first aid manual includes information on the emergency treatment of accidents as well as the more common illnesses. The clarity of presentation makes it a good text for first aid trainees in civil defense or other emergencies. A chapter deals with poisoning, but suggestions for first aid are in general terms only. Physical first aid procedures are well illustrated and a general index is included.

Tests and Standards for New and Nonofficial Remedies 1955 (Supplement to Tests and Standards 1953). Edited by WALTER WOLMAN. The American Medical Association, 535 North Dearborn St., Chicago, Ill., 1955. ix + 161 pp. 20 x 12.5 cm. Price $2.

This volume is a supplement to *Tests and Standards for New and Nonofficial Remedies 1953* and contains all tests and standards monographs for nonofficial products that have appeared in *New and Nonofficial Remedies* since 1953. Thus, the present volume includes tests and standards for nonofficial products accepted by the Council on Pharmacy and Chemistry under its former seal of acceptance program, between January 1, 1953, and January 1, 1955. In addition, many monographs that have been extensively revised since 1953 also appear in this *Supplement*.

The format and style of the *1955 Supplement to Tests and Standards for N. N. R. 1953* are essentially the same as in the original book. The monographs described commercial materials actually manufactured for use in pharmaceuticals, and as a consequence physical characteristics given for a basic drug or an active ingredient of a dosage form may differ from those reported in the literature for highly purified materials. Most physical properties and tests for purity are determined by the techniques described in detail in U. S. P. XV. A notable exception to this rule, however, is the determination of melting point by a method different from that prescribed by the official compendia. In some instances more than one method of assay is given for the determination of a characteristic group or molecule because the equipment required for one method may not be available to all laboratories. As far as possible the assay procedure for an active ingredient of a dosage form is adapted from the method given for the basic drug.

Like the official compendia, the term "water" means distilled water, "solution," without any other designation, means a solution in water, the abbreviation "C" is no longer used following temperature designations, and several other conventions employed in the U. S. P. and N. F. are followed.

In *Tests and Standards for New and Nonofficial Remedies 1955* will be found definitions, structural formulas, physical descriptions, tests for identity and purity, methods of assay for the active ingredients of dosage forms, and tolerance limits for those drugs accepted by the Council on Pharmacy and Chemistry for which official standards are not available. This contribution from the American Medical Association Chemical Laboratory is of great value to pharmaceutical manufacturers and others with a special interest in standards for new drugs and their dosage forms. Dr. Wolman and his associates are to be commended for the excellence of this latest compilation of tests and standards which previously have been checked or developed in the American Medical Association Laboratory. It is hoped that this program of the A. M. A. Laboratory will be continued.

BOOKS RECEIVED

Directory American Council of Independent Laboratories, Inc. 6th ed. Waverly Press, Inc., 1956. xxxi + 67 pp. 17.5 x 26 cm.

1956 Medical Progress. By MORRIS FISHBEIN. McGraw-Hill Book Company, Inc., New York, 1956. x + 389 pp. 15.5 x 24 cm.

Study Guide for Pharmacy Jurisprudence. By WILLIAM R. ARTHUR. 4th ed. Hemphill's, Texas, 1956. 74 pp. 22.5 x 28 cm. Price $3.25.

Product Liability Cases. By FRANK T. DIERSON and CHARLES WESLEY DUNN. Commerce Clearing House, Inc., Chicago, 1955. xlvii + 1134 pp. 17.5 x 25.2 cm. Price $12.

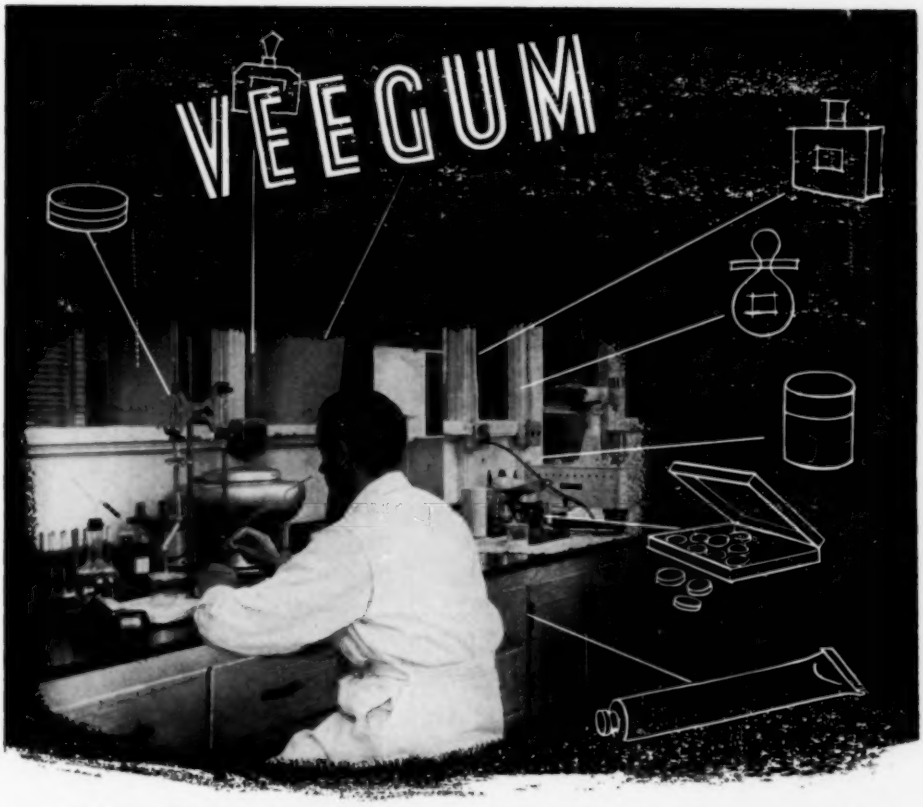
Actualités Pharmacologiques. Published under the direction of RENÉ HAZARD. Masson et Cie., Paris, 1955. 198 pp. 16.5 x 25 cm. Price 1,480 fr.

Handbook of Medical Library Practice. 2nd ed. Edited by JANET DOE and MARY LOUISE MARSHALL. American Library Association, Chicago, 1956. xv + 601 pp. 16 x 24.5 cm. Price $10.

Accidents Thérapeutiques en Dermatologie. Published under the direction of JACQUES CHARPY. Masson et Cie., Paris, 1955. 342 pp. 16 x 24 cm. Price 2,000 fr.

Administration of Medical and Pharmaceutical Patents. By ARCHIE M. PALMER. National Academy of Sciences—National Research Council, Washington, D. C., 1955. 69 pp. 17.5 x 25 cm. Price $2.

Alcoholism—Its Psychology and Cure. By FREDERICK B. REA. The Philosophical Library, Inc., New York, 1956. 143 pp. 12.5 x 19 cm. Price $3.50.



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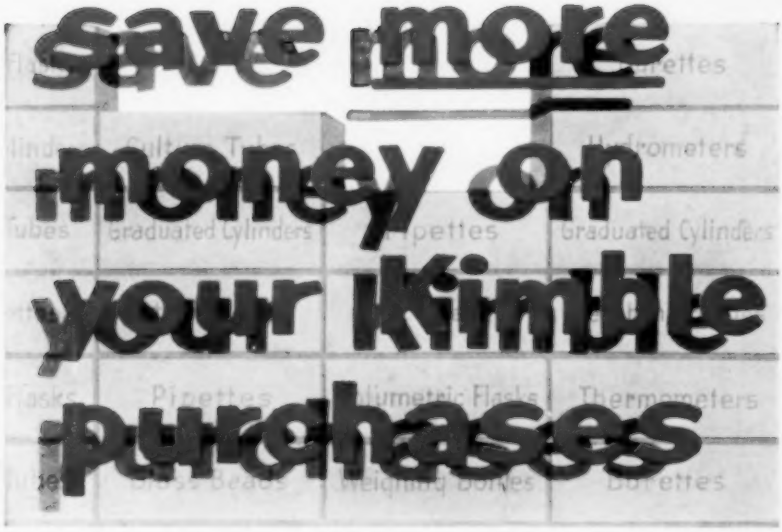

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Recently new lower prices became generally effective on thermometers and hydrometers. So, now you may get even greater savings by including Exax thermometers and hydrometers with the rest of your Kimble apparatus.

THERMOMETER MARKINGS ARE PERMANENT

The filler used in the lines and numbers of Kimble thermometers is permanent—it can be removed only by dissolving the glass itself.

INDIVIDUALLY RETESTED

Every Kimble thermometer and hydrometer is *Individually Retested* before shipping to insure accuracy. N.B.S. specifications are minimum standards for Kimble thermometers and hydrometers. There is also a line of Kimble instruments made to A.S.T.M., and A.P.I. and M.C.A. specifications.

Your local laboratory dealer can supply these fine instruments at new lower prices. Do not accept substitutes. If your dealer will not supply Kimble hydrometers and thermometers, write us. Kimble Glass Company, subsidiary of Owens-Illinois, Toledo 1, Ohio.

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Improved manufacturing facilities and increased production make new lower prices possible on Kimble thermometers and hydrometers.

Description and Catalog Number	Range	Qty. in case	Each	1 case	5 cases	10 cases	25 cases
Thermometer #46260-Y Aniline Point	−30 to 42°C						
Thermometer #45504 Laboratory Grade	−20 to 110°C						
Hydrometer #31302 Specific Gravity	1.045 to 1.105 Sp. gr.						
Hydrometer #31634 API	10 to 31 API°						

SAVE MORE by ordering these items with your other Kimble glassware requirements. Most laboratory dealers offer greater discounts on case quantity purchases.

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